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Through the Lifecycle

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HYPOXIA

Through the Lifecycle

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PREFACE

The International Hypoxia Symposium convenes biannually to bring together international experts from many fields to explore the state of the art in normal and pathophysiological responses to hypoxia. Representatives from five continents and 32 countries joined together in February 2003 for four days in the dramatic mountains of Banff, Alberta.

As editors of the Proceedings of the International Hypoxia Symposia, we strive to maintain a 26 six year tradition of presenting a stimulating blend of clinical and basic science papers focused on hypoxia. Topics covered in 2003 include hibernation and hypoxia, hypoxia and fetal development and new advances in high altitude pathophysiology, oxidative stress and membrane damage, hypoxic regulation of blood flow, heat shock proteins in hypoxia, and future directions in hypoxia research.

In 2003 we also had the privilege of honoring John W. Severinghaus as a friend, colleague, mentor and inspiration to many in the field. Tom Hornbein's personal tribute to John Severinghaus is the first chapter in this volume, followed by an entertaining update of the history of the discovery of oxygen written by John Severinghaus.

A tribute by Ken Storey to our late friend and colleague, Peter Hochachka, is in Chapter 23. Peter was a longtime supporter of the International Hypoxia Symposia, and a dear personal friend; he is greatly missed!

Another role for the International Hypoxia Symposia is to serve as an international forum for a variety of working groups focused on particular problems, usually related to high altitude physiology or pathophysiology. The International Hypoxia Symposia are home to the Lake Louise Acute Mountain Sickness (AMS) Score; the Children's AMS Score and the Guidelines for Children at High Altitude. Continuing that tradition we present a paper from the chronic mountain sickness (CMS) working group. A new CMS score is presented, and the first English translation of the classic paper on CMS and its scoring, "La desadaptacion a la vida en las grandes Alturas", is presented along with the original article in Spanish. Another working group met at the International Hypoxia Symposia present in Chapter 25 their proposal for a standard approach to AMS epidemiology.

The abstracts from the 2003 meeting were published in High Altitude Medicine and Biology 3(4), 2002, with several late abstracts presented in Chapter 26.

We hope that this collection of papers especially prepared for this volume allows us to share with a broader audience some of the intellectual excitement that embodies the spirit of the Hypoxia meetings.

Robert C. Roach, Peter D. Wagner, Peter H. Hackett, Editors, May 2003.

ACKNOWLEDGMENTS

The 13th International Hypoxia Symposium was a rewarding experience due to the outstanding faculty and the lively participation of our largest group of participants. At this, our third meeting as the organizers, we were especially pleased that the experience known as the Hypoxia Meetings can continue to prosper. We remain always thankful for the kind and wise guidance of Charlie Houston, the originator of the Hypoxia meetings.

Ms. Joann Bauer of the University of Colorado Continuing Medical Education office provided professional support and kept everything running smoothly so we could focus on the science. Thanks Joanne!

In 2003 we had the generous support of a number of organizations and individuals, including the U.S. Army Research and Development Command, The White Mountain Research Station and Drs. Luciano Bernardi and Robert Schoene. And thanks are also due to numerous others who freely gave of their time and energy to make the meeting such a resounding success.

Please join us by the light of the full moon in February 2005 for the 14th International Hypoxia Symposium.

Robert Roach and Peter Hackett, Chairmen
International Hypoxia Symposia (www.hypoxia.net)

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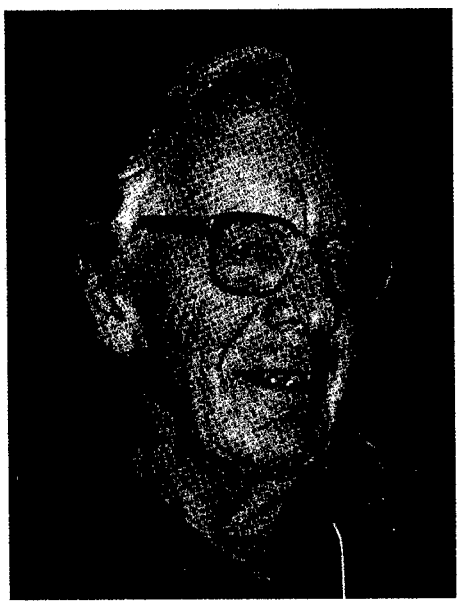
Chapter 1

A TRIBUTE TO JOHN WENDELL SEVERINGHAUS

Thomas F. Hornbein



I remember too well the nervousness I felt four decades ago as John was teaching me to perform my first jugular bulb puncture. Our team was setting out to measure what happens to brain blood flow in lowlanders newly arrived at the Barcroft Laboratory on White Mountain, an altitude of 12,500 feet. I was doing my best to appear cool as John's words guided the needle I held into the jugular bulb...into *his* jugular bulb. Oh, and I should not forget the preamble to this learning experience, John's account of the prior time this procedure had been performed on him, by Mr. Cerebral Blood Flow himself, the late Niels Lassen *(Editor's Note: See reference (23) for a Tribute to Niels Lassen by John Severinghaus).* In the punchline of that account, John recalled the several subsequent days he enjoyed a numb tongue, thanks to Niels' needle nailing a nerve.

This, the initial exploration of what happens to cerebral blood flow upon ascent of lowlanders to high altitude, was sequel to John's first sojourn to the Barcroft Lab a few years earlier. On that occasion John's question was whether ventilatory acclimatization to hypoxia could be explained by compensatory readjustments of the pH in the fluid surrounding brainstem chemosensors, as reflected (hopefully) by the pH in the spinal fluid sampled from the lumbar space in each of the subjects, a.k.a. investigators. John, of course, was the chosen, the one gifted with a week-long headache as a result of the spinal puncture.

The publication resulting from this headache built upon the earlier discovery by Bob

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

Mitchell, Hans Loeschke, and Severinghaus of the presence of CO_2/H^+ sensitive chemosensor cells near the ventrolateral surface of the cat's medulla. This, John's first White Mountain study proved to be a pivotal event, stimulating years of challenging, important research not only from John's lab but by a good many other seasoned scientists, attempting to prove (or disprove) the monarchy of the hydrogen ion as the central regulator both of ventilation and cerebral blood flow.

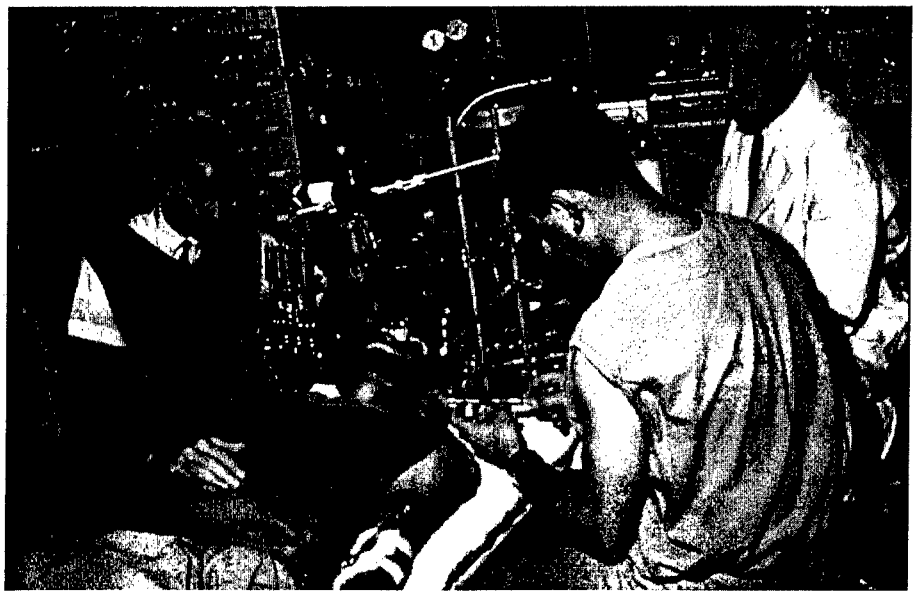


Figure 2. Photo of John supervising jugular bulb needling of TFH.

My second scientific journey with John took place a few years later. We were off to the altiplano of Peru, I to quantify chemoreceptor activity in high altitude cats, John and Soren Sorenson to assess the sluggish ventilatory responsiveness to hypoxia of high altitude humans. There was a precious albeit breathless loveliness to living for some weeks at Cerro de Pasco (4300 m), a colorful mining community at the same height as my hometown mountain, Mt. Rainier.

The chemical regulation of ventilation and brain blood flow were where John's and my professional interests most closely intersected. John's insatiable curiosity has stirred the pot in two other high altitude arenas: 1) what causes the leak in HAPE, and 2) how best to assess the ventilatory response to hypoxia.

Severinghaus and Whayne were the first to seek an animal model for HAPE by causing rats to swim (to avoid drowning) in an oxygen-deficient atmosphere. John proposed that the leak of fluid into interstitial and alveolar spaces might occur upstream from constricted pulmonary arterioles because of over-distention by high pressure in the elastic pulmonary arteries.

Figure 3. John W. Severinghaus and Thomas F. Hornbein working hard at Cerro de Pasco, Peru.

Regarding the hypoxic ventilatory response (HVR), Severinghaus and Bainton in 1964 demonstrated the diminished HVR of high altitude dwellers in South America, particular those whose high hematocrits qualified them for a diagnosis of chronic mountain sickness or Monge's Disease. Sorensen and Severinghaus then showed that this insensitivity to hypoxia persists for years after natives of high altitude moved to sea level, and also after cardiac surgery repaired the holes in the hearts of blue babies. And at this conference, now four decades later, John is still working on a consensus of the best methods for measuring HVR.

High altitude and its consequences are but a piece of the vast scope of John's inquiry. He began residency training measuring the uptake by the body of inhaled nitrous oxide, a "first". He introduced computerized systems to continuously monitor the anesthetic gases and oxygen and CO_2 in all the anesthetized patients in a suite of operating rooms, developed methods for transcutaneous measurement of oxygen and carbon dioxide, standardized the human oxygen dissociation curve and discovered an accurate equation to describe it, leading to his recent fascination with the evaluation of pulse oximeter performance.

Though by no means John's greatest scientific discovery, in my opinion, his greatest gift to the world is his and Freeman Bradley's bringing the electrode measurements of PO_2, PCO_2 and pH to what is now referred to by clinicians everywhere as ABGs, arterial blood gases. The events preceding this evolution began with Richard Stow's 1954 invention of a PCO_2 electrode by wrapping a wet pH electrode with a rubber glove. Severinghaus added the crucial chemical change, making it stable and doubling its sensitivity. After Leland Clark invented his oxygen electrode, using a polyethylene membrane between blood and a platinum electrode, John and Freeman Bradley proceeded to package pH, PCO_2 and PO_2 electrodes into a Plexiglas, temperature-controlled water bath, the 1957 prototype of

which now resides in the Smithsonian Museum in Washington D.C. Having electrodes that would enable a clinician to characterize respiratory and acid-base perturbations from small amounts of blood in a short period of time was, to me, a seminal "aha" (a favorite John expression). First, this ability to monitor blood gases in near real time transformed the practice of anesthesiology from an art form where the major focus had been on rendering a patient unconscious to a practice of acute care medicine, where assessing and responding to moment-to-moment changes in a patient's physiology became possible. These seeds of monitoring, planted first in the operating room, soon sprouted a whole new discipline, intensive care medicine. Only occasionally does one person's vision have such a huge impact on the rest of the world. For this one, we have John and his box to thank.

John Wendell Severinghaus was born in 1922, grew up in Madison, Wisconsin, obtained a BS in physics from Haverford College, and spent WWII developing radar at MIT. While there, he espied a lovely young Wellesley student, Elinor Peck, and immediately realized he was destined to marry her. She took a little longer to come to the same realization. When the A bomb dropped, John dropped physics and within a week was admitted to medical school. He split his medical education between the University of Wisconsin in his hometown and the College of Physicians and Surgeons, Columbia University, New York. Elinor finally said yes and they were married in August 1948. John interned in Cooperstown, N.Y., then on to Philadelphia for residency training in anesthesiology with Robert Dripps along with a postdoctoral fellowship with Julius Comroe. Three productive years at the N.I.H. Clinical Research Center, where he served as director of anesthesia research preceded the completion in 1957 of his anesthesia training in Stuart Cullen's esteemed department at the University of Iowa. When Comroe was recruited to UC San Francisco to found the Cardiovascular Research Institute, he invited John to join him, whereupon John persuaded Comroe to recruit Cullen to found the anesthesia department there. Cullen had no trouble persuading John to follow his two favorite mentors to UCSF in mid 1958.

I cannot do justice here to the breadth and depth of John Severinghaus's wide-ranging inquiry over nearly half a century but will conclude by reflecting upon John and his environmental impact upon those of us whose paths have had the fortune of crossing his.

- John is a glutton for punishment, who will do unto himself before doing unto others. Biomedical research contains a noble history of such seekers who test their theories on themselves first.
- John's eclectic curiosity about how things work, and why, transcends that of most of us normal humans. His questioning has carried him into realms too numerous to elaborate in this brief bit; his affection for high altitude is but one. His touch has been felt in many domains of medicine and science.
- His curiosity comes coupled with an ingenuous questioning to which some of us have been exposed at scientific meetings over many decades. In his early years, his simple need to understand could feel intimidating to a young recipient of his questions. John's style has ripened to a nurturing grace that makes him a most special mentor, guide, and catalyzer, especially for young investigators. He gives of himself generously and with obvious pleasure.
- All these things and more add up to a joy of discovery that is the essence of a great adventurer and an infectious gift to those who would follow where he has led.

As I write this, John is in his 80th year. These latter years have for him, as for so many of us, slowed the body and at times challenged the soul. But his spirit sings loud and lovingly to those whose lives he has touched. A piece of that refrain is heard in one of John's favorite Grooks by the Danish poet/philosopher, Piet Hein:

I'd like to know
what this whole show
is all about
before it's out

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Chapter 2

FIRE-AIR AND DEPHLOGISTICATION

Revisionisms of oxygen's discovery

John W. Severinghaus

Abstract: Americans are taught that Joseph Priestley discovered oxygen in 1774 and promptly brought that news to Lavoisier. Lavoisier proved that air contained a new element, oxygen, which combined with hydrogen to make water. He disproved the phlogiston theory but Priestley called it dephlogisticated air until his death 30 years later.

Scandanavians learn that a Swedish apothecary Carl Wilhelm Scheele beat Priestley by 2 years but was deprived of credit because Lavoisier denied receiving a letter Scheele later claimed to have sent in September 1774 describing his 1772 discovery of "fire air". His claim was unconfirmed because Scheele first published his work in 1777. However, Scheele's missing letter was made public in 1992 in Paris, 218 years late, and now resides at the French Academie de Sciences. Lavoisier received it on Oct 15, 1774. His guilt was kept secret in the effects of Madame Lavoisier. He failed on several occasions to credit either Priestley or Scheele for contributing to the most important discovery in the history of science. Priestley was a teacher, political philosopher, essayist, Unitarian minister and pioneer in chemical and electrical science. He discovered 9 gases including nitrous oxide. He invented soda water, refrigeration, and gum erasers for which he coined the term "rubber". He discovered photosynthesis. He was humorless, argumentative, brilliant and passionate, called a "furious free-thinker". While his liberal colleagues Josiah Wedgwood, Erasmus Darwin, James Watts, and others of the Lunar Society were celebrating the 2nd anniversary of the French revolution, a Birmingham mob, supported by the royalists and the established church, destroyed Priestley's home, laboratory and church. Driven from England, he emigrated to Pennsylvania where he built a home and laboratory and collected a 1600 volume library, then among the largest in America. He is regarded as a founder of liberal Unitarian thinking. He was friend and correspondent of Thomas Jefferson. His philosophy and insight persuaded Jefferson to initiate what Americans call a liberal arts education. Scheele was later recognized as a brilliant and productive pioneer in chemistry although he died at age 44 of tasting his own arsenic compounds. In the new time-lapse play "Oxygen" set in Stockholm in both 18th and 21st centuries, in 1774, blame falls on Lavoisier's wife who hid Scheele's letter in hopes of giving her husband sole credit for discovering

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Kluwer Academic/Plenum Publishers, New York, 2003.

oxygen. In 2001, four Nobel committee panelists cannot agree which should receive the first "Retro-Nobel Prize" for the greatest discovery of all time: Priestley, Scheele or Lavoisier or all three. The audience is asked to choose.

Key Words: Priestley, Scheele, Lavoisier, Arcadia, Roald Hoffman, Carl Djerassi, phlogiston

INTRODUCTION

The more elaborate our means of communication, the less we communicate

-Joseph Priestley

Scandanavians are taught that Swedish apothecary Carl Wilhelm Scheele generated oxygen in Uppsala in 1772, although his publication is dated 1777. Americans are taught that the English Unitarian minister and chemist Joseph Priestley discovered oxygen on August 1, 1774 and personally informed Lavoisier in Paris in September 1774. Lavoisier gradually realized this gas was a new element, which he named oxygen. He overturned the conventional phlogiston theory. It has been called the most important discovery in the history of chemistry.

Why does controversy about priority remain? Scheele claimed (in his 1777 book) that he wrote Lavoisier in September 1774 describing his 1772 experiments. Lavoisier repeatedly denied seeing or receiving that letter. Without the letter, it was difficult to maintain that such a letter was ever sent or received.

Priestley's claim is supported by contemporary documentation and has never been in doubt. Indeed, he wrote much in protest about Lavoisier having misappropriated his work. Despite this, some historians believe Lavoisier didn't need Priestley's disclosure.

My interest in these historic events was stimulated by an invitation to give the Joseph Priestley lecture at Penn State Medical School in Hershey, PA. With that came a visit to Priestley's restored home and laboratory in Northumberland far up the Susquehanna River where he settled in the 1790's. The Priestley House is now a National Monument. It was the site of formation of the American Chemical Society in 1874, the centenary of oxygen's discovery.

The American and English Anesthesia History Associations, meeting in Bristol 4 years ago, visited Priestley's 1774 laboratory in Calne, Wiltshire, southwest England. The location is Bowood, a romantically landscaped park and garden with Greek pavilion and a hermitage, designed by Capability Brown shortly before Priestley's time there. Lord Byron had been a guest there about 1809, seduced another guest's wife, a duel may have occurred, after which Byron hurriedly left England.

Bowood appears to be the real life model of the fictitious Sidley Park, the setting of Tom Stoppard's "Arcadia". That fascinating play jumps between 1809 and the present in the library of a great house which is undergoing picturesque revisions of Capability Brown's romantic designs. The play features both a Greek pavilion and a hermitage. Byron has just left after a fatal duel with a jealous husband. The cause of his flight occupies two competing 20th century literary historians who are plumbing the library for juicy details. In 1810, the precocious 15-year-old Tomasina Coverly tells her tutor Septimus one should be able to write an equation for a rose. In despair at not being betrothed by her 15th birthday,

she unsuccessfully tempts Septimus. After her suicide, he spends the rest of his life in the hermitage scribbling equations, trying to grasp her invention of fractals.

There are other historical parallels to the play. Byron married Annabelle Milbanke, English mathematician, an associate of Charles Babbage. Their daughter, Lady Augusta Ada Byron, born in 1815, was the mathematician who wrote the world's first computer program for Charles Babbage's computer, and after whom the computer language ADA is named. Perhaps Arcadia's young polymath Tomasina Coverly is modeled after the historic Ada Byron.

PRIESTLEY (1733-1804)

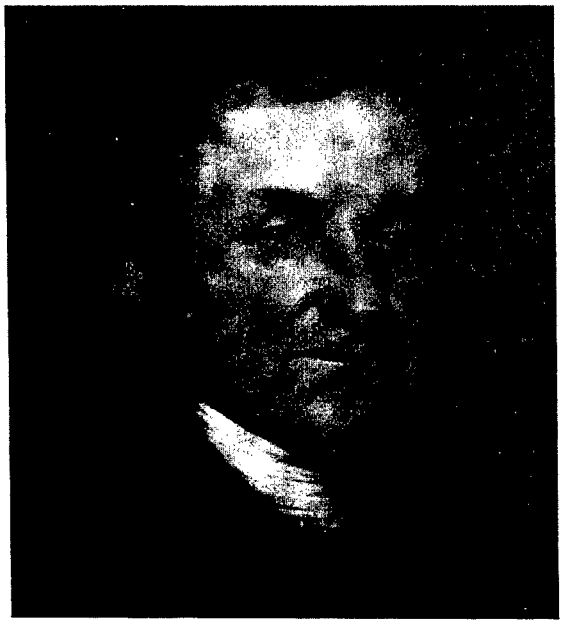

Figure 1. Joseph Priestley (1733-1804) by Gilbert Stuart, ca1801.

Born near Leeds in 1733, Joseph Priestley (Figure 1) was the oldest of six children of a modestly successful cloth dresser (4). His Calvinist parents sent him to study theology at a new Dissenting Academy at Daventry, Northamptonshire. He found history, philosophy, and science more interesting than theology. In 1755 he became assistant minister to a Presbyterian congregation in Needham Market, Suffolk. Priestley's beliefs matured from Calvinism to rational Unitarianism. His unorthodox and even heretical opinions as a "furious freethinker" gradually lost him the confidence of his orthodox congregation, and he resigned.

In 1758 he transferred to a more sympathetic (anti-establishment) congregation in Nantwich, Cheshire, where he opened a day school with 36 students. He taught science,

obtaining an air pump and a static generator for electrical demonstrations.

In 1762 Priestley married Mary Wilkinson, aged 18, the sister of one of his students. Her father, Isaac Wilkinson, an iron master at Bersham, Denbigh, in Wales developed the accurate way of boring true cylinders for James Watt's steam engines, pumps and cannon. His firm still exists as the Wilkinson Sword Company. His marriage provided Priestley with means to be an amateur scientist. The Priestleys had a daughter and three sons.

He then taught at Warrington Academy, near Liverpool. Because Oxford and Cambridge Universities and the learned professions were closed to Dissenters, Priestley developed new courses and textbooks that were suitable for students preparing for careers in industry and commerce. His 'Rudiments of English Grammar' remained in use for 50 years. He made Warrington Academy the most distinguished school of its kind in England. Edinburgh University in 1765 conferred an honorary doctorate on him. His thesis was 'Eminent men of all ages'.

In 1765, Priestley met Benjamin Franklin, with whose encouragement and generous loan of the requisite books, he published The History and Present State of Electricity, including his own experiments. He discovered that charcoal conducts electricity and noted the relationship between electricity and chemical change. In 1766 Priestley was elected to membership in the Royal Society.

In 1767 Priestley became pastor of the Mill Hill Chapel in Leeds, a free church where his home was next to a brewery. By dissolving the CO_2 produced by fermentation in water he generated cheap soda water, then called windy water, to avoid importing spa waters from France. This won him the Copley medal of the Royal Society in 1773. He began chemistry experiments, and discovered 4 new gases at Leeds: Nitrous air (NO), red nitrous vapor (NO_2), diminished nitrous air (N_2O, later called laughing gas) and HCl. However, even in the Leeds Free Church, the fury of his anti-establishment preaching led to dismissal.

In 1773, the Earl of Shelburne employed him as librarian, literary companion, and tutor to his two young sons at Bowood, with the freedom to preach and write as he wished. On August 1, 1774, he discovered that, by heating the mineral red mercuric oxide in a sealed glass chamber, using a newly acquired burning glass, a new gas was liberated in which a candle burned furiously. He showed that a mouse could live longer in it than in a similar sealed volume of air. He discovered photosynthesis by showing that a sprig of mint left in air in which a mouse had died, regenerated the substance needed to keep a mouse alive. Unable to discard his chemical education based on the phlogiston theory, he called this new gas 'dephlogisticated air'. To fit the old theory he proposed that phlogiston had negative weight.

Priestley's earlier report of the nitrous airs led to correspondence with Antoine Laurent Lavoisier, France's most distinguished chemist. In September, 1774, Lord Shelburne accompanied Priestley to Paris where he described his methods and discoveries to a distinguished group of French scientists at Lavoisier's home. It is still disputed whether that visit was the critical spark of Lavoisier's revolution of chemistry.

Priestley as a Furious, Free Thinking Liberal Philosopher

Priestley published violently anti-establishment essays, books and pamphlets some of which were seen even by supporters as dangerously controversial. In his History of

the Corruptions of Christianity Priestley rejected most of the fundamental doctrines and traced them to their historical sources of error. This work aroused a storm of protest. Lord Shelburne, William Fitzmaurice-Petty, later Marquis of Lansdowne, was very much part of the establishment. It was he who later negotiated the treaty ending the American Revolution. After the Earl's second marriage, Priestley's writings were an embarrassment. They parted company in 1779.

Priestley moved to Birmingham as minister of the wealthy, intellectual dissenting New Meeting congregation. He joined the Lunar Society with Josiah Wedgewood (pottery), Erasmus Darwin (physician and Charles' grandfather), James Watts (steam engine), Matthew Boulton (iron and steel manufacturing), Dr. William Withering (digitalis), Richard Lovell Edgeworth (inventor), chemist James Keir, geologist John Whitehust, Samuel Galton, a Quaker gun maker and William Small, Professor of Natural Philosophy. They met on Monday night near each full moon to facilitate the horseback homeward trip. Outsiders called it the lunatic society.

Priestley became a major political theorist among 18th-century liberals, and a major mind of the Enlightenment. He founded and edited the first Unitarian journal. As a follower of Locke, he emphasized individualism. He believed that people should have a voice in their government and power over their own actions. He coined the government policy motto: "The greatest happiness of the greatest number". He espoused both the American and French revolutions. He fought for Parliamentary reform and against the legal and civil impediments of dissenters.

By the time of the French Revolution he was regarded as a threat to church and state. His outspoken rebuttal of Edmund Burke's attack on the French revolution made him exceedingly unpopular with the predominantly royalist public. He became the prime target for political cartoons, editorial denunciation and threats.

On July 14, 1791, while the Lunar Society celebrated the second anniversary of the French revolution, a royalist mob, reputed to have been encouraged by local clergy, ransacked Priestley's laboratory. His instruments, books and papers were destroyed. The mob violence spread immediately, burning his church, laboratory and home, another non-conformist church and other homes. Priestley escaped in disguise on horseback. For three years Priestley taught at New College, Hackney, London. In 1793, when England and France went to war, Priestley had to leave England. He and his wife sailed to America, joining his sons.

He settled briefly in Philadelphia, then America's capitol, influenced by his friendships with Benjamin Franklin, Thomas Jefferson and John Adams. He declined offers from New England and New York of professorships and a ministry. To escape the avaricious Quakers and Yellow Fever, he designed and built his home on the Susquehanna River in Northumberland, speculating that it would become Pennsylvania's capitol. A college that Priestley was to have headed failed because the legislature disagreed with Priestley's liberal religious ideas.

He preached in Philadelphia during Thomas Jefferson's inauguration. After hearing him several times, Jefferson wrote him, 'Yours is one of the few lives precious to mankind for the continuance of which every thinking man is solicitous.' His correspondence with Jefferson altered the course of higher education toward the American liberal arts college curriculum.

Although Priestley was a self-taught chemist, he invented gum erasers for which he

coined the term 'rubber', discovered the electrical conductivity of charcoal and carbon and was the first to discover photosynthesis. He described how to use compressed liquefied gases to produce refrigeration. He established the first scientifically equipped laboratory in the United States, and published over 150 papers and books, which fill about 28 volumes. Before Priestley's work, only three gases were known: air, carbon dioxide, and hydrogen. Priestley discovered 9 gases: NO, N_2O, NO_2, O_2, SO_2, HCl, SiF_4, H_2S, and NH_3. His success resulted in large part from his ability to design ingenious laboratory apparatus. His pen never stopped. Seven months before his death he described himself in a letter to a friend as 'an exhausted volcano'. He died in Northumberland Feb. 6, 1804. His was the quintessential Enlightenment mind of a great communicator.

CARL WILHELM SCHEELE (1742-1786)

Figure 2. Carl Wilhelm Scheele (1742-1786). This is a symbolic post mortem painting, modified by J. Falander from a well-known portrait of Goethe.

Scheele (Figure 2) was born on December 9, 1742, one of eleven children. He received little formal education and no scientific training. At age 14, he was apprenticed to apothecaries in Gothenburg, Malmö and Stockholm. He read the scientific books of the day and started experimenting.

In 1770, he moved to Uppsala as a laboratory assistant under Sweden's great chemist Torbern Bergman. While there, Scheele discovered 'fire air' [oxygen], probably in 1771. He produced this new gas using at least 4 different chemical reactions. He demonstrated that common air consists of fire air, which supports combustion, and foul air (nitrogen),

which does not. His book, On Air and Fire, also describes Scheele's experiments with hydrogen sulfide gas, which he was the first to synthesize. Scheele noted the action of light on chloride of silver and the insolubility of blackened silver chloride in ammonia - discoveries that would later prove significant for photography.

How then was it that Scheele failed to publish his discovery for almost 6 years? Two clear reason stand out. First, because he was unable to interpret his experiments in terms of the phlogiston theory, he failed to understand how important a discovery it was. And second, he apparently wished to put all his discoveries together in a book instead of publishing separate papers. Lavoisier, knowing of Scheele's earlier work, had sent Scheele a copy of his book in the spring of 1774. Scheele claimed that he had written to thank Lavoisier on September 30, 1774, describing ways of preparing fire air and asking Lavoisier to repeat them with his larger burning lens. Lavoisier never replied and later denied having seen the letter, which would have established Scheele as the true discoverer of oxygen.

In early 1775, Bergman mentioned Scheele's discovery in a Latin science report (1), which was not rediscovered for 2 centuries. Scheele's book was ready for the press in December, 1775, but its publication was delayed because Bergman did not deliver his promised preface until July, 1777. In some reports this delay is blamed on the printer, in others on Bergman's suspected jealousy. By the time of publication of On Air and Fire in 1777, Scheele had learned of Priestley's discovery, and of Lavoisier's subsequent confirmation and reinterpretation of chemical theory. It was too late to claim priority.

However, significantly, on February 4, 1775, Scheele was elected to membership in the Swedish Royal Academy of Sciences. This great honor (with the King of Sweden in attendance) had never before (and never since) been given to a student of pharmacy. Clearly his great discoveries had been recognized in Sweden, almost certainly before his purported letter to Lavoisier in September 1774. Unfortunately, no document is known describing his work at that event.

In 1775, Scheele moved to Köping, Sweden. Because of his royal honor, the town provided him his own pharmacy where he took a position as superintendent, declining several academic positions. He wrote: "Oh, how happy I am! No care for eating or drinking or dwelling, no care for my pharmaceutical business, for this is mere play to me. But to watch new phenomena, this is all my care, and how glad is the enquirer when discovery rewards his diligence; then his heart rejoices"

Scheele investigated compounds of cyanide and arsenic. Without analytic methods, he tasted the poisons he made. He was aware that this was the cause of his poor health. He referred to it as "the trouble of all apothecaries." He died, probably from arsenic poisoning, at age 43 on May 26, 1786. He is now credited with the discovery of 7 elements (N, O, Cl, Mn, Mo, Ba, W) and many compounds: HF, SiF, H_2S, HCN, glycerol, tartaric acid, citric acid, lactic acid, uric acid, benzoic acid, gallic acid, oxalic acid, lactose, prussic acid, arsenic acid, molybdic acid, and tungstic acid. His copper arsenite (called "Scheele's green") was used to decorate candy for 50 years before it was found to be a poison! Due to his humility, many of his discoveries were reported either too late or were made public by Bergman without Scheele's consent and were incorrectly credited to others.

ANTOINE LAURENT LAVOISIER (1743-1794)

Figure 3. Antoine Laurent Lavoisier (1743-1794)

Lavoisier (1743-1794; Figure 3) was a versatile genius, primarily a chemist, but also a statesman, financier, economist, manufacturer and landowner (3). He had life long interests and significant accomplishments in stratigraphic geology, scientific agriculture, political reform, government finance and humanitarian social reform. He was born in Paris to a newly rich lawyer with peasant ancestors, educated in science, literature, philosophy and law in the best schools, and exposed to chemistry by the flamboyant popularizer Rouelle. Abandoning his legal degree, he tackled geology first, then chemistry. Lavoisier was 28 when he married the 13-year-old Marie Anne Pierrette Paulze (1759-1836; Figure 4), daughter of his professional colleague and close friend Jacque Paulze.

In 1774 he and several French chemists were working with the red calyx of mercury, and with the curious discovery that, upon heating, it was restored to metallic liquid mercury, evolving a gas he assumed was Black's fixed air, CO_2. Before Priestley's visit, Lavoisier had been made a member of a committee of the Academy of Science to investigate the red calyx and its gas. According to biographer Henry Guerlac at Cornell (2), Lavoisier did not need Priestley's disclosure to discover oxygen.

The conventional historical view is that, following Priestley's visit, Lavoisier repeated the experiment successfully. He soon realized that this gas and the mercury weight loss were not compatible with the phlogiston theory. He understood that the evolved gas explained the loss of weight of the heated red mercury. And he soon realized that it was an element present in atmospheric air, which combined with fuel to make fire.

Lavoisier repeatedly denied having received Scheele's letter although it must have arrived at the time of Priestley's visit. Priestley promptly published his discovery of the new gas he called dephlogisticated air, keeping Lavoisier informed by mail of his ongoing experiments.

However, inexplicably, nine months after Priestley's description of this new gas, Lavoisier published his "discovery" that the evolved gas was not Black's fixed air but a new gas that made flame burn brightly. He wrote and spoke at the Royal Academy of Priestley's experiments as if he had done them himself, and without giving credit to either Priestley or Scheele. He first called the new air 'eminently breathable air', but in 1777, he named it oxygen, incorrectly believing it was a component of all acids (oxy in Greek is sharp, or acid). For at least 6 years after naming oxygen, he continued to call it vital air.

For more than a decade many chemists remained skeptical that this gas was an element rather than dephlogisticated air. Lavoisier was able to overcome the old ideas using precise quantitative measures of volume and weight of the reactants. Henry Cavendish had reported years earlier that moisture appeared when hydrogen (which he discovered) was burned with air. Lavoisier repeated the Cavendish experiment in 1783 and announced that water was a compound of hydrogen and oxygen. This was the final nail in phlogiston's coffin. The discovery of oxygen coupled with his brilliant insights revolutionized chemistry.

Figure 4. Lavoisier and his wife, Marie-Anne Pierrette Paulze Lavoisier (1759-1836). Painted about 1788 by the famed French artist Jacques Louis David on commission from Mm. Lavoisier. (Metropolitan Museum, NY)

OXYGEN

A new play, 'Oxygen' was authored by Roald Hoffman, Professor of Chemistry at Cornell who received the chemistry Nobel prize in 1982, and Carl Djerassi, the Stanford biochemist and writer who popularized the pill for contraception nearly 40 years ago (Figure 5).

Figure 5. Prof. Carl Djerassi (Chemistry, Stanford) and Prof. Roald Hoffman (Chemistry, Cornell, Nobel Prize 1982) at the premiere of their new play Oxygen, San Diego, April 2, 2001 with the author.

Their play's thesis is that a committee in Stockholm, in 2001, proposes awarding the first 'Retro-Nobel Prize' for the greatest discovery of all time to celebrate the centenary of the Nobel Prize. Their choice is oxygen. The Nobel committee's discussions are interwoven with flashbacks to 1777 (curiously similar to those of Arcadia).

The protagonists, Priestley, Scheele and Lavoisier and their wives are invited to Stockholm by Gustav III in order to choose whose contribution was most important. When they each claim the major credit, the king decides not to make an award. The four members of the 2001 Nobel committee also take four different views of who really to credit for oxygen's discovery, three choosing a different man, and the chair choosing all three. They cannot agree and do not award a Retro-Nobel Prize. A central event in the play is the rediscovery by a clever PhD candidate, in Madame Lavoisier's necessaire, of the missing letter from Scheele to Lavoisier, establishing Scheele's priority and Lavoisier's guilt.

THE LONG LOST LETTER RECOVERED

Scheele's missing letter has, in fact, been recently rediscovered and made publicly available! It had been claimed to be seen in the 1890's by a French chemist and historian,

E. Grimaux. He wrote 'Une lettre in Èdite de Scheele a Lavoisier' in Revue generale des sciences pures et appliquees, 1890, Vol 1, p1-2. He stated that he had found that letter in a collection of papers and artifacts of Lavoisier's wife. He described it and published the text. However, the letter was never made available to historians or scientists. It appears to have again been hidden, leading to disbelief among historians, and casting doubt on Scheele's claim.

Amazingly, that original letter finally came to light in 1992 in a donation to the Archives de l'Acadèmie des Sciences, Lavoisier Collection, in Paris from the holders of Mme Lavoisier's artifacts. To date there has been little scholarly work on it, and on the descendants' reasons for keeping it secreted for 218 years. This strange story lends support to the conjectures of 'Oxygen' about the ethics of the Lavoisiers. I learned of its existence through the play, obtained photographs of it from Professor Hoffman, and found it a highly relevant topic to present to today's scientists.

There remains no doubt about the priority of Scheele's discovery of oxygen. Priestley is usually credited with making the same discovery available to others, especially Lavoisier. Historians assume that Lavoisier did receive Scheele's letter on Oct 15, 1774, probably one week after discussing similar work directly with Priestley. Guerlac (3) asserts that 'In any case, Lavoisier seems to have been too preoccupied with other matters to follow up Scheele's suggestion'. While neither Priestley nor Scheele understood their discoveries, Lavoisier gradually understood how this overthrew all the theories of fire and respiration. It is not improbable that Lavoisier wished to be credited for such a discovery, which only he understood. And as his understanding progressed, the magnitude of the discovery must have loomed temptingly before him. It was, we now see, the most important discovery in the history of science. He coined the word oxygen in 1777 still thinking it was a component of all acids but continued to refer to it as eminently breathable air and later as vital air for nearly another decade.

What is history's final judgment on Lavoisier's integrity? Was it Lavoisier's purpose to claim discovery of oxygen? His subsequent statements often suggested so. Other scientists accused him on several occasions of failure to acknowledge other's work, attempting to claim their discoveries as his own. Priestley repeatedly wrote pamphlets and letters in which he accused Lavoisier of borrowing his ideas, and of making claims for experiments done by Priestley, not himself. Edmond Genet wrote that Lavoisier had read to the Academy, as his own work, a restatement of a letter Genet had written to Lavoisier describing Priestley's experiments. Between 1772 and 1777, Priestley's publisher Magellan wrote 13 letters through M. Trudaine to Lavoisier describing Priestley's research. Despite this, on March 26, 1775, Lavoisier announced at the Academy that he had identified the part of atmospheric air that was more specific to respiration, without reference to Priestley.

HISTORIC REVISIONISM IN THEATER

Why did Lavoisier, usually so punctual in his correspondence, fail to respond to Scheele? Was he too preoccupied? The authors of 'Oxygen' suggest a fascinating and plausible alternative idea, that: **Madame Lavoisier hid the letter from her husband.**

They suggest that Marie-Anne wanted her husband to get the credit for the discovery of oxygen. She was clever, multi-lingual, mathematically able, a trained draftsman and

became his secretary, bookkeeper and laboratory assistant. She handled his mail, and probably did receive and read Scheele's letter shortly after the visit from Priestley. As her husband's laboratory assistant and partner, she may have conceived the idea of not disclosing the letter to him to help him achieve the fame of the new discovery.

The historic materials belonging to Madam Lavoisier included a 'necessaire', a book-like box containing small things she would need, such as sewing materials, pen, ink, paper, and a mirror. This box was sold to Cornell University in 1993, and photos of it play an important role in 'Oxygen', as the location in which Scheele's missing letter had been hidden behind a broken mirror (Figure 6). The play includes an imaginary letter from Madam Lavoisier to her husband as he awaits the guillotine, asking his forgiveness for having secreted the letter.

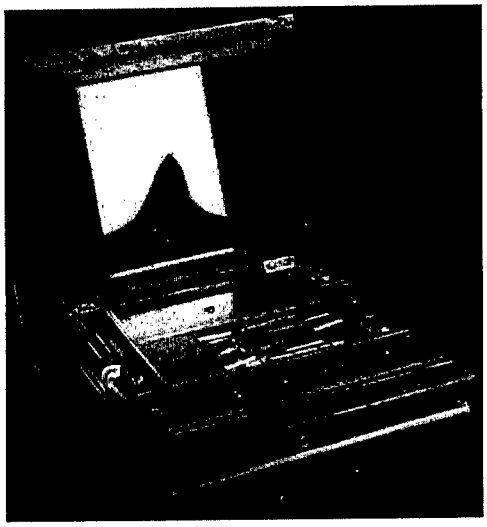

Figure 6. Mm. Lavoisier's 'Necessaire' with broken mirror, now at Cornell University. The fictitious location of Scheele's lost letter in the new play Oxygen.

THE LESSONS FOR SCIENTISTS

The axiom 'Publish or Perish' certainly applies to the uncommunicative apothecary Scheele. He loved chemical experimentation but cared little about making a name or fortune. Priestley was a scholar, a 'furious free thinker', a great communicator, the 'High Priest of the Enlightenment" (4), and a scientist. He published his every observation and thought. His mind was flexible enough to overthrow much of Christian doctrine, but not of phlogiston. Scheele was a great chemist who discovered 7 elements and a host of compounds but failed to promptly add them to the body of knowledge and thereby lost credit for much of his work. Neither Priestley nor Scheele ever believed Lavoisier's theory that their gas was an element, oxygen. Lavoisier was a brilliant economist, entrepreneur, chemist and meticulous scientist. His mind was flexible enough to reject all prior chemical

theory based on a few simple observations. But in the oxygen discovery it seems he lacked professional integrity.

The Retro Nobel Prize committee begs us to choose the awardee. My choice is all three. Each was flawed but had a quality needed in science, Scheele's imaginative experimenting, Priestley's curiosity, instrumental abilities and facility with writing and speaking and disclosing his work, and Lavoisier's facile insight and thorough preparation. Oxygen needed its three discoverers each using his own talents.

Why was Scheele's letter secreted for 218 years, and then quietly deposited in the archives of the French Academie de Sciences as the world was consumed with the war against Iraq? Few then noticed that France had finally admitted that their most illustrious chemist was guilty of perhaps the most significant example of scientific misconduct in history.

This paper was adapted and extended from a previous paper on the subject by the author (5).

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Chapter 3

MAMMALIAN HIBERNATION
Transcriptional and translational controls

Kenneth B. Storey

Abstract: Mammalian hibernation is an amazing strategy for winter survival. Animals sink into a deep torpor where metabolic rate is <5% of normal, body temperature falls to 0-5°C, and physiological functions are strongly suppressed. Hibernation is a closely regulated process that includes multiple controls on gene transcription and protein translation, the primary subjects of this review. Recent studies by our lab and others have used multiple techniques of gene discovery, including cDNA array screening, to identify genes that are up-regulated in hibernation and continuing studies are tracing the functions of the encoded proteins and the signal transduction systems that regulate expression. For example, up-regulation of fatty acid binding proteins during hibernation facilitates the switch to a primary dependence on lipid fuels by nearly all organs and new studies have shown that up-regulation is mediated by the PPARγ transcription factor and its co-activator, PGC-1. Several hypoxia-related genes including HIF-1α are also up-regulated during hibernation suggesting a role for this transcription factor in mediating adaptive responses for hibernation. Controls on mRNA translation during hibernation accomplish two goals: a general strong suppression of protein synthesis that contributes to energy savings and the selected synthesis of a few specific proteins. These goals are accomplished by mechanisms that include reversible phosphorylation controls on ribosomal initiation and elongation factors and differential distribution of individual mRNA species between polysome and monosome fractions. Studies of gene expression, protein synthesis regulation, controls on fuel metabolism, and signal transduction pathways are combining to produce an integrated model of the biochemical regulation of hibernation.

Key Words: metabolic rate depression, gene expression, cDNA arrays, signal transduction, fatty acid binding protein

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

INTRODUCTION

Hibernation is the key to winter survival for many small mammals. By strongly suppressing metabolic rate, falling into a deep torpor and letting body temperature (Tb) drop to near ambient hibernators can save as much as 90% of the energy that would otherwise be needed to maintain euthermia (Tb ~37°) throughout the winter (31). During hibernation all body functions are suppressed to low levels. For example, ground squirrels hibernating with a core Tb of 5°C show a heart rate of only 5-10 beat per minute compared with the euthermic value of 350-400 beats per minute. Breathing drops from greater than 40 to less than1 breath per minute and breathing patterns in many species can include long periods of apnea, ranging from minutes to hours (21). Metabolic rate in hibernation (at a Tb of 0-5°C) is typically only 1-5 % of the corresponding resting rate in euthermia. The biochemical and physiological mechanisms that regulate hibernation are fascinating and exploration of these is not only key to understanding this amazing phenomenon but may also illuminate answers to applied problems in human health such as how to improve the hypothermic preservation of mammalian organs removed for transplant (24) or how to limit atrophy during long periods of skeletal muscle disuse.

Our studies of hibernation began because of our interest in the biochemical mechanisms that control metabolic rate depression. Our work with a variety of other systems (anoxia tolerant turtles and molluscs, estivating toads and snails) had indicated that the principles of metabolic arrest were conserved across phylogeny and included a central role for reversible protein phosphorylation in coordinating the suppression of multiple enzymes and functional proteins in order to re-establish cellular homeostasis at a new and much lower net rate of ATP turnover (25,26). In hibernator systems it soon became apparent that these same principles applied. Reversible protein phosphorylation plays a key role in coordinating metabolic suppression during hibernation and in reorganizing metabolic priorities for sustained function in the hypometabolic state. For example, tissue-specific reversible phosphorylation of selected enzymes of glycolysis (glycogen phosphorylase, phosphofructokinase, pyruvate kinase) as well as pyruvate dehydrogenase, the enzyme that gates carbohydrate entry into the Krebs cycle, is key to sparing carbohydrate catabolism during hibernation in order to favor lipid oxidation (23,25). The activities of energy-expensive ion-motive ATPases (Na^+K^+ATPase, Ca^{2+}ATPase) are also suppressed by this mechanism as are key functional proteins involved in ribosomal translation (26). These reversible controls on key loci allow metabolic functions to be rapidly and coordinately suppressed and then re-activated during each arousal period for hibernation is not continuous throughout the season but consists of multiple bouts of deep torpor lasting 1-3 weeks interspersed with brief arousals of up to a day. Our current interests in hibernation focus on three areas: (1) signal transduction pathways that mediate both metabolic and gene expression changes during hibernation, (2) the role of differential gene expression in hibernation, and (3) the differential controls on protein translation that provide a strong net suppression of protein synthesis while at the same time allowing synthesis of selected key proteins. The remainder of this article focuses on these areas under two general headings - transcriptional control and translational control - with related aspects of signal transduction integrated into each section.

TRANSCRIPTIONAL CONTROL IN HIBERNATION

Hibernation is a seasonal phenomenon with a circannual rhythm that is reinforced by photoperiod cues. It is supported by a large suite of metabolic adaptations, acting over different timeframes, most of them requiring changes in gene expression. Some metabolic adjustments occur long before hibernation commences such as the accumulation of huge reserves of body lipids (body mass can increase by >50%) during a late summer period of hyperphagia. Supporting this, activities of fatty acid biosynthesis enzymes peak at this time but are then depleted and replaced with increased activities of enzymes of fatty acid catabolism as the hibernating season begins. Proliferation of brown adipose tissue (BAT) also occurs pre-hibernation; this thermogenic organ provides animals with the means to reheat their bodies and escape from each torpor bout. Other adjustments are made once the hibernating season begins and may be triggered during a series of "test drops" in Tb that occur prior to the first excursion into deep torpor. Others may be activated (or renewed) as the animal sinks into each hibernation bout.

Recent studies by our lab and others have used diverse techniques of gene discovery to identify a variety of hibernation-responsive genes. In ground squirrels these have included α_2-macroglobulin in liver (22), moesin in intestine (13), isozyme 4 of pyruvate dehydrogenase kinase (PDK4) and pancreatic lipase in heart (1), isoforms of uncoupling protein (UCP) and fatty acid binding protein (FABP) in multiple tissues (4,15), the ventricular isoform of myosin light chain 1 ($MLC1_v$) in heart and skeletal muscle (10), organic cation transporter 2 in kidney (17), the melatonin receptor (20) and four genes on the mitochondrial genome: NADH ubiquinone-oxidoreductase subunit 2 (ND2), cytochrome c oxidase subunit 1 (COX1) and ATPase subunits 6 and 8 (10,16). Notably, a substantial number of hibernation-responsive genes have been found in heart (Figure 1). This may be related to the fact that heart must continue to work during torpor and adjustments in gene expression are undoubtedly necessary to optimize cardiac muscle function with respect to the changes in Tb, work load and fuel availability that occur in torpor.

Although the genes identified to date represent a wide assortment of cellular proteins, some principles of adaptation are beginning to emerge and will provide interesting directions for new studies over the next few years. For example, the up-regulation of $MLC1_v$ in ground squirrel heart, when combined with studies of hamster heart that show changes in the proportions of myosin heavy chain isoforms during hibernation, suggests that myosin restructuring occurs during hibernation. This would presumably provide an optimal mix of myosin isoforms to adapt the contractile apparatus to the new work load and thermal conditions of the torpid state. Other studies suggest that adjustments must be made to minimize the risk of thrombosis in the microvasculature under the very low blood flow (ischemic) conditions of the hibernating state. The observed up-regulation and export of α_2-macroglobulin (a protease inhibitor that affects proteases of the clotting cascade) from the liver (22) along with reduced platelet numbers (sequestered into the spleen) and reduced levels of several clotting factors all support a decreased clotting capacity during torpor (20). Another direction of great interest to us stems from the observation that several mitochondrially-encoded genes, coding for subunits of mitochondrial proteins, are up-regulated in hibernation. This is significant because when we evaluated transcript levels of related nuclear-encoded genes (e.g. subunit 4 of COX and ATPα, a nuclear-encoded subunit of the mitochondrial ATP synthase), these did not change during hibernation (Figure 1) (16).

Interestingly, up-regulation of genes encoded on the mitochondrial genome also occurs in freeze tolerant and anoxia tolerant animals (26) so this may be a general response by stress-tolerant animals that could have important, but as yet unknown, adaptive significance.

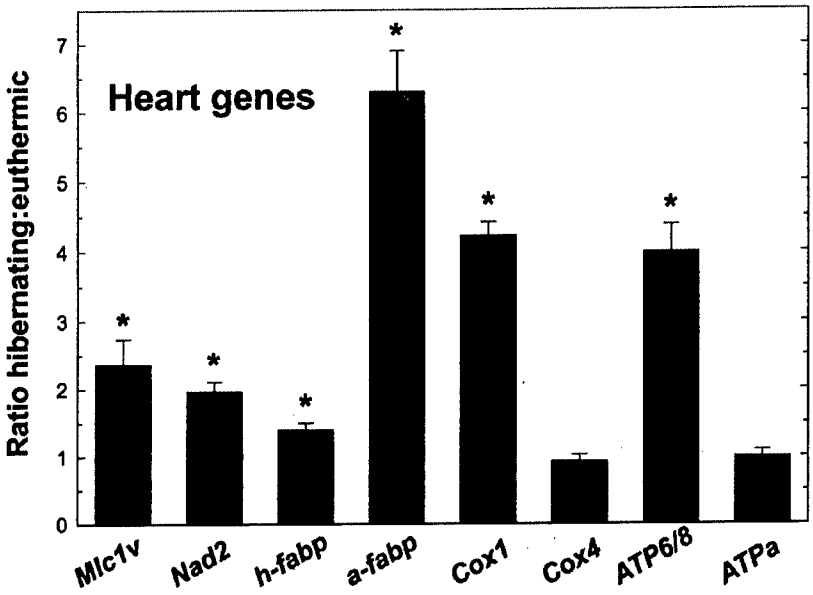

Figure 1. Gene response to hibernation in ground squirrel heart. Relative transcript levels in hearts from hibernating versus euthermic animals were determined from northern blots for eight genes: *Mlc1*$_v$, myosin light chain 1 ventricular isoform; *Nad2*, subunit 2 of NADH-ubiquinone oxidoreductase; *h-fabp* and *a-fabp*, heart and adipose isoforms of fatty acid binding protein; *Cox1* and *Cox4*, subunits 1 and 4 of cytochrome c oxidase; *ATP6/8*, ATPase 6/8 bicistronic mRNA; and *ATPα*, the alpha subunit of the mitochondrial ATP synthase. *Nad2*, *Cox1* and *ATP6/8* are encoded on the mitochondrial genome and whereas *Cox4* and *ATPα* are nuclear-encoded subunits of mitochondrial proteins. Data for *MLC1*$_v$ and *Nad2* are from *Spermophilus lateralis* (10); others are from *S. tridecemlineatus* (15,16). Data are means ± S.E.M., n=3; * - transcript levels are significantly higher in hibernator, compared with euthermic, heart, $P<0.05$.

Gene Screening With cDNA Arrays

The genes identified to date probably represent only a fraction of the total hibernation-responsive genes but one new method of gene discovery, cDNA array screening, should soon remedy that and our recent experiences with this technique deserve some commentary. State-of-the-art glass slide microarrays can now have up to 31,500 non-redundant cDNAs bound to them and their use provides the opportunity for simultaneous screening of the responses by thousands of genes to a single stress. Hence, the opportunities for gene discovery by this technique are tremendous. In particular, array screening has two key assets: (1) the opportunity to identify genes (and thereby implicate pathways or functions) that are key to hibernation but have never before been considered as participating in the

phenomenon, and (2) the opportunity to evaluate the responses to hibernation by functionally-related groups of enzymes/proteins such as the series of enzymes involved in a MAPK signal transduction pathway or the family of mitochondrial membrane transporter proteins. For example, screening of skeletal muscle extracts from the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, showed that several genes that encode components of the small and large ribosomal subunits were down-regulated during hibernation including L19, L21, L36a, S17, S12 and S29 (9). This implicates control of the ribosomes as critical to the inhibition of protein synthesis in hibernation (see section on translation control below).

One potential concern with the use of array screening is the issue of heterologous probing for, to date, arrays have been produced for only a handful of model species. In our recent studies we have used ATLAS™ nylon macroarrays containing cDNAs for 588 rat genes (Clontech) and human 19,000 cDNA microarrays (Ontario Cancer Institute) to screen for hibernation-specific gene expression in ground squirrels and bats (Figure 2) (9,15). Obviously, there are gene sequence differences between species and therefore cross-species hybridization between a rat or human gene array and a sample prepared from ground squirrel or bat mRNA will not be perfect. This would be a problem if the goal was to assess the responses to hibernation of selected specific genes but instead we have used arrays as a tool for general gene discovery. In this mode, we are looking for genes that are differentially expressed, by 2-fold or more, between control (euthermic) and experimental (hibernating) states. Used in this mode with multiple tissues from ground squirrels and bats, array screening has provided us with dozens of putatively up-regulated genes representing multiple cell functions, enough to keep the lab busy for several years to come. Indeed, we found that cross-hybridization between mammalian species was actually very high. Our first studies with rat macroarrays showed 93% cross-hybridization between the cDNA fragments on the array and *S. tridecemlineatus* cDNA samples. Comparable studies with tissue samples from little brown bats, *Myotis lucifugus*, showed 73% cross-hybridization. With the human 19K microarrays some optimization of hybridization and washing conditions was needed but we were then able to achieve 85-90% hybridization which allowed us to assess the responses to hibernation by over 16,000 genes (9).

Our results to date from the use of nylon macroarrays to search for hibernation-responsive genes have provided several new insights that are currently being pursued. For example, analysis of liver and kidney samples from both ground squirrels and bats were consistent in indicating up-regulation of genes associated with antioxidant defense during hibernation. Compared with the euthermic state, differential screening indicated a significant up-regulation (>2-fold) of glutathione-*S*-transferase, glutathione peroxidase and superoxide dismutase in kidney. These same enzymes as well as peroxiredoxin and metallothionein showed >2-fold up-regulation in liver. It is well known that hibernators elevate their antioxidant defenses in BAT as a means of dealing with high rates of oxygen free radical generation during thermogenesis (6) but our gene screening data suggests that the improvement of antioxidant defenses also occurs in other tissues. This would aid organs in their defense against oxyradical damage during the arousal process when oxygen consumption of all tissues can rise by 10-20 fold within minutes as the animal rewarms to 37°C. Interestingly, these new data on hibernators fit well with our previous studies on antioxidant defenses in anoxia-tolerant and estivating animals and show that enhancement of antioxidant defenses is a universal response to stresses that involve wide variation in oxygen availability or consumption (14). Array screening also showed putative up-regulation

of several heat shock proteins in liver (Hsp40, Hsp60, Hsp70) during hibernation whereas results for kidney showed pronounced increases in transcript levels of transport proteins: a 2-fold increase in aquaporin 3, a 5-fold increase in sodium-proton exchanger isoform 2, and a 7-fold rise in organic cation exchanger isoform 2 (*Oct2*). As will be discussed later under translational controls in hibernation, the up-regulation of *Oct2* (and perhaps of the other transporters) does not lead to an immediate increase in OCT2 protein but appears instead to be an anticipatory response that elevates transcript levels in order to allow rapid synthesis of the protein during the arousal period.

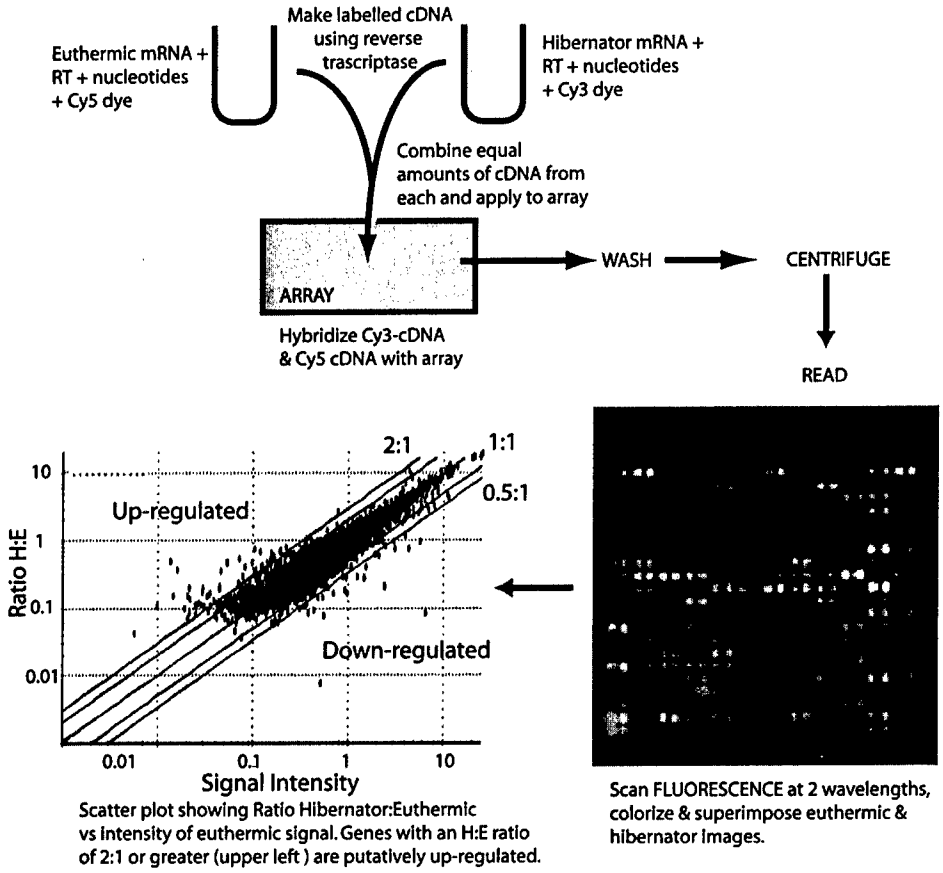

Figure 2. The steps involved in cDNA array screening and a typical scatter plot showing the ratio of gene expression in skeletal muscle from hibernating (Cy3-labeled) vs euthermic (Cy5-labeled) *S. tridecemlineatus*. Genes showing an H:E ratio of >2 are considered as putatively up-regulated. Note that the vast majority of gene transcripts are unaffected during hibernation, with H:E ratios between 0.5 and 2.0; only a very few genes are specifically up- or down-regulated during hibernation.

Fatty Acid Binding Protein, PPARs, PGC-1 and Akt

Our first use of the ATLAS™ rat macroarrays probed hibernation-responsive gene expression in ground squirrel brown adipose. Until recently, studies of hibernator BAT have focused primarily on the process of mitochondrial thermogenesis and, in particular, on the regulation and action of UCP1. However, there are other aspects to thermogenesis, a key one being fuel supply and array screening provided us with a starting point for several new studies that are exploring the regulation of fuel metabolism in hibernators. One prominent result from array screening of BAT was the very strong up-regulation of the adipose (A) and heart (H) transcripts of *fabp* during hibernation (15). Subsequent analysis by northern blots using cDNA probes retrieved from a ground squirrel BAT cDNA library confirmed a 2-3 fold up-regulation of mRNA levels for both transcripts in BAT as well as up-regulation of both isoforms in heart and of *h-fabp* in skeletal muscle. Increased transcript levels correlated with elevated FABP protein during hibernation (17).

The presence of both isoforms of FABP in BAT and heart has not been reported previously in mammals and may be an adaptive feature for hibernation. The two isoforms are believed to have different functions in fatty acid transport. A-FABP is believed to carry fatty acids to and from intracellular lipid droplets. Because it can form a complex with hormone-sensitive lipase, it has been suggested that a major function is to carry fatty acids away from lipid droplets after triglyceride hydrolysis. In white adipose this transport would mainly be to the plasma membrane for export whereas in BAT transport to the mitochondria would be the probable destination (30). By contrast, the role of H-FABP is to pick up incoming fatty acids at the plasma membrane and transport them to the mitochondria. The presence of both isoforms in hibernator BAT reflects the fact that the tissue uses both its own internal lipid reserves and fatty acids imported from white adipose tissue to fuel nonshivering thermogenesis. The primary role of H-FABP in heart is to transport incoming fatty acids from the sarcolemma to the mitochondria for under normal conditions the mammalian heart derives ~70% of its energy from lipid oxidation. The unusual induction of *a-fabp* in heart of hibernating ground squirrels (transcripts were absent from euthermic heart) may be related to unique circumstances in hibernators. The hearts of hibernators are unusual among vertebrates in that they maintain substantial intracellular triglyceride lipid droplets (5). These are probably needed to meet the demand for high rates of ATP output from fatty acid oxidation during arousal, a demand that could exceed the capacity for triglyceride delivery via the blood at the low Tb values that characterize the early phase of arousal. Hence, expression of A-FABP in hibernator heart provides the organ with access to both intracellular and extracellular lipid reserves. A role for A-FABP in low temperature function is also supported by the fact that the only other reported instance of A-FABP presence in heart was in hearts of Antarctic teleost fishes (29). Hence, lipid-based heart metabolism at low temperatures may be aided by the maintenance of triglyceride reserves within the cardiomyoctes and the presence of A-FABP. Interestingly, our analysis of the H-FABP sequence from ground squirrels showed that this protein also has modifications that could aid low temperature function. Three unique amino acid substitutions place polar amino acids in positions that are filled by non-polar or hydrophobic amino acids in human or rat sequences (15). These occur in positions that would alter the flexibility of the protein and may contribute to the effective function of H-FABP at low temperatures.

The up-regulation of FABP in hibernator organs led us to examine the signal transduc-

tion systems that could be involved in this response. In new studies with bats, *M. lucifugus*, we used Western blotting to analyze the responses during hibernation of transcriptional activators of fatty acid catabolism, the gamma isoform of the peroxisome proliferator-activated receptor (PPARγ), a ligand-activated transcription factor, and its co-activator, PGC-1 (Figure 3) (S. Eddy and K. Storey, manuscript submitted for publication).

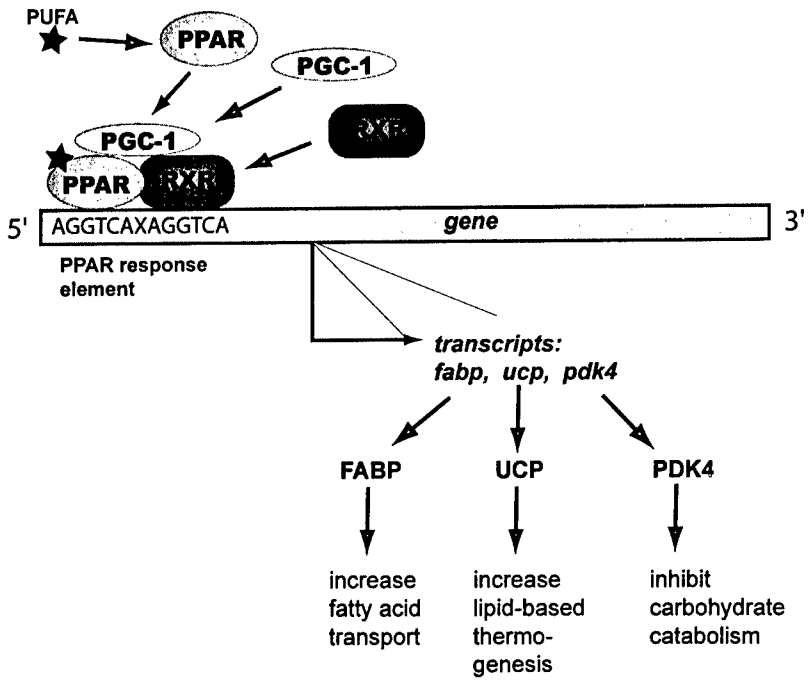

Figure 3. When activated by the binding of a ligand such as a polyunsaturated fatty acid (PUFA), peroxisome proliferator-activated receptors (PPAR) form heterodimers with retinoid X receptors (RXR) and bind to the PPAR response element (AGGTCAXAGGTCA; where X is a variable base) of selected genes. Binding is enhanced/stabilized by the PPAR co-activator, PGC-1. Binding activates transcription of selected genes. In hibernators, these probably include genes for fatty acid binding protein (FABP), mitochondrial uncoupling protein (UCP) and pyruvate dehydrogenase kinase 4 (PDK4).

All genes involved in lipid catabolism are thought to contain a PPAR response element (PPRE) to which PPAR isoforms bind as a heterodimer complex with activated retinoic acid receptors; this is potentiated by a number of activating factors, one of which is PGC-1 (3). Figure 4 summarizes our results for BAT and skeletal muscle. As also occurred in ground squirrels (15), *M. lucifugus* BAT displayed elevated amounts of mRNA transcripts and protein for both A- and H-FABP during hibernation whereas skeletal muscle showed elevated H-FABP only (heart and skeletal muscles have the same isoform). In both organs FABP up-regulation was correlated with increased levels of PPARγ and PGC-1 protein. Indeed, PPARγ and PGC-1 levels showed parallel increases during hibernation in four

other organs as well (heart, liver, kidney, white adipose) but both decreased in brain. The bats under study had been hibernating for about 36 h after a 12 h euthermic interval so it could be proposed that elevated PPARγ and PGC-1 in bat organs stimulated renewed gene expression and protein synthesis of selected proteins that are key for survival in torpor and/or for the next arousal period, proteins that may have been depleted or damaged during the euthermic interval. PPARγ is known to increase the expression of A-FABP in other mammals and two other known targets of PPARγ in mammalian adipose are PDK4 and the mitochondrial uncoupling protein (3). As mentioned earlier, both are up-regulated during hibernation (1,4). Thus, we have strong evidence that proteins involved in promoting lipid oxidation are up-regulated in a coordinated fashion under the control of the PPAR transcription factors during hibernation. Furthermore, it is interesting to note that the gamma isoform of PPAR is typically described as being abundant in adipose tissue and low in other tissues of non-hibernating mammals (3). However, this is not true in bats as the transcription factor was found in all seven tissues tested. This may attest to an enhanced importance of PPARγ and PGC-1 in the regulation of fatty acid catabolism in hibernating species and may represent an adaptive modification of a signal transduction pathway to play a specific role in hibernation.

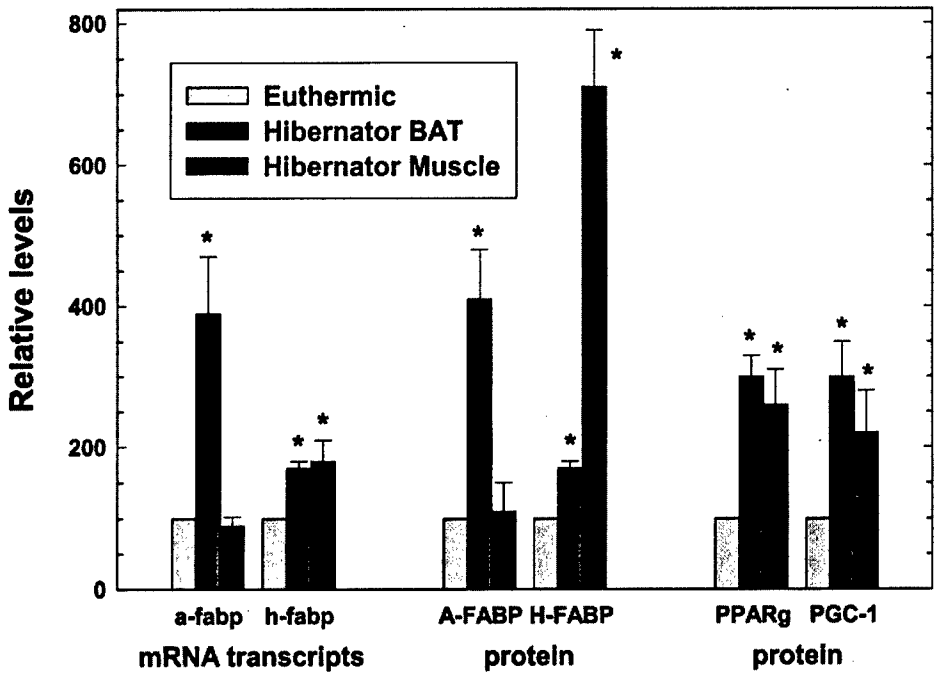

Figure 4. Effect of hibernation (36 hour re-entry into torpor) on transcript and protein levels of the adipose (A) and heart (H) isoforms of fatty acid binding protein and protein levels of the transcription factor, PPARγ and its co-activator, PGC-1, in brown adipose tissue and skeletal muscle of bats, *M. lucifugus*. Transcript levels were measured by Northern blotting; proteins by Western blots. Data are means ± S.E.M., n=3; * - values are significantly higher in hibernator, compared with euthermic, tissues, $P<0.05$.

PPAR-mediated up-regulation of genes/proteins involved in fatty acid oxidation is a well-known response in the starved state in all mammals and, clearly, as far as fuel use is concerned, hibernation is basically a long-term starvation. PPAR effects are opposed by the insulin signaling pathway which in the fed state stimulates glucose uptake into cells and its storage as glycogen or use as a substrate for fatty acid biosynthesis. Indeed, transcription of PGC-1 in mammals is inhibited by insulin and this is one of the regulatory mechanisms that provide reversible control over fatty acid biosynthesis versus oxidation. We wondered then about the status of the insulin signaling pathway during hibernation. One of the elements of this pathway is Akt (also called protein kinase B) which has multiple functions. For example, activation of Akt is linked with increased glucose uptake into muscle cells probably via stimulation of the glucose transporter, GLUT4. Akt also promotes glycogen synthesis by phosphorylating and inhibiting glycogen synthase kinase 3 which prevents the enzyme from inactivating glycogen synthase. We assessed Akt responses during hibernation using Western blotting with two kinds of antibodies - one that detects total Akt protein and one that is specific for the peptide containing the phosphorylated residue, phospho-Akt being the active form. During hibernation, phospho-Akt content in *M. lucifugus* organs was reduced or unchanged in six organs with particularly strong suppression in liver and white adipose (total Akt was also reduced in white adipose). These data indicate reduced insulin signaling in hibernation and the reorientation of fuel metabolism to favor lipid oxidation. Therefore, it appears that hibernators employ well-known mammalian regulatory mechanisms to control fuel consumption over the extended months of dormancy.

Hibernators and Hypoxia

The organs of hibernating mammals are hypoperfused and, assessed by the standards of an active mammal at 37°C, would be considered to be severely ischemic; for example, cerebral blood flow is only ~10% of the euthermic value (reviewed in 20). Some researchers have argued that hibernators would make good models for studying ischemia. For selected facets of ischemia, this is probably true. For example, hibernators can provide an excellent model for assessing solutions to the problem of blood clot formation in the microvasculature at low flow rates and studies to date have noted a number of significant adjustments to hibernator blood that lower clotting capacity during torpor (reviewed in 20). However, as a model system for studying resistance to hypoxia damage, a hibernator model is probably not a good one. The oxygen content of hibernator blood may be lower than that of the euthermic animal but metabolic rate is also 20-100 fold lower than in euthermia. Furthermore, there are no metabolic indicators of oxygen-limitation during hibernation. Lactate does not build up and the respiratory quotient remains at ~0.7, indicative of aerobic lipid oxidation and consistent with the depletion of body lipid depots over the winter months. In addition, the hibernator in deep torpor clearly retains the capacity to supply sufficient oxygen to brown adipose to support the massive lipid-fueled thermogenesis required for arousal. So, although apnoic breathing patterns may mean that blood oxygen content varies over a considerable range during torpor, it is not likely that the organs of the animal are ever oxygen-limited.

Having said this, there are now at least two lines of evidence that indicate that, in some manner, hypoxia has a role to play in hibernation. These are:

(1) A ancient hypoxia-hypothermia interaction may contribute to the mechanism of

metabolic rate depression in hibernation. Hypoxia leads to a drop in Tb in many mammalian species and hibernating species show a more pronounced drop in Tb than do nonhibernators. Using ground squirrels in the summer season, Barros *et al.* (2) found that under hypoxia metabolic rate was not simply suppressed but was regulated to assist the initial fall in Tb and then acted to stabilize Tb at a new lower level. Indeed, a new set point was established for Tb as long as hypoxia persisted. However, oxygen was not limiting in this situation since a drop in ambient temperature caused the animals to elevate their metabolic rate to maintain the new Tb (this also occurs in hibernation if ambient temperature falls below 0°C). Hence, it is possible that hypoxia signals (perhaps generated from breath-hold episodes) may contribute to initiating and managing the drop in metabolic rate and Tb that occurs during entry into torpor.

(2) Hibernating animals show up-regulation of hypoxia-related genes. Our use of cDNA arrays to screen for hibernation-responsive genes has consistently shown positive responses by hypoxia-related genes in heart and skeletal muscle. These include putative up-regulation during hibernation of HIF-1α, HIF-1β (or ARNT), ORP150 (oxygen regulated protein) and proline hydroxylase. We are presently exploring the expression patterns of these genes and their proteins to try to understand what role they may be playing. In other systems, HIF is well known as an inducer of glycolytic enzymes (32) but this does not seem to be the case in hibernators. We surveyed the activities of glycolytic enzymes (glycogen phosphorylase, hexokinase, phosphofructokinase, aldolase, pyruvate kinase, lactate dehydrogenase) in seven organs of *Spermophilus richardsonii* and found 3 instances of somewhat higher lactate dehydrogenase activities in hibernating animals but no consistent pattern of glycolytic enzyme enhancement during hibernation (M. de la Roche and Storey, unpublished results). Hence, it does not appear that a HIF signal is elevating the glycolytic potential of organs during hibernation and it remains to be determined what the HIF signal is doing.

TRANSLATIONAL CONTROL IN HIBERNATION

Another area of active current research in hibernation is translational control. Protein synthesis is one of the major energy expenditures of cells, requiring ~5 ATP equivalents per peptide bond formed and consuming as much as 40% of the total ATP turnover in selected organs such as liver. Not surprisingly, then, a strong reduction in the overall rate of protein synthesis is an integral part of metabolic rate depression in hibernation. For example, studies with ground squirrel brain have show that the rate of ^{14}C-leucine incorporation into protein *in vivo* during hibernation was only 0.04% of the value in euthermic squirrels (11). Part of this rate suppression was due to the difference in Tb between the two states (37°C vs 7.5°C) but when brain extracts were assessed *in vitro* at a constant 37°C, the rate of protein synthesis in hibernator extracts was just 34% of that in euthermic extracts (11). Similar measurements in kidney extracts *in vitro* showed that the hibernator rate was just 15% of the euthermic value but, interestingly, protein synthesis by brown adipose tissue extracts was not suppressed during torpor (17). Inhibition of protein synthesis during hibernation might arise from (1) reduced mRNA substrate availability, and (2) a stable inhibition of the ribosomal translational machinery. Substrate availability is a factor in any metabolic process and mRNA limitation undoubtedly affects the production of selected proteins but,

overall, there is little, if any, change in global mRNA levels or transcript levels of various constitutive genes during hibernation (11,26). Evidence from cDNA array screening of ground squirrel or bat tissues also shows that mRNA transcript levels for most genes are unaffected during hibernation (9,15). Thus, during hibernation, existing mRNA transcripts are effectively maintained in storage, with a greatly extended lifespan, and are held in readiness for the resumption of protein synthesis upon arousal.

Regulation of Initiation and Elongation

New studies are showing conclusively that translational control during hibernation comes from specific inhibition of the ribosomal machinery. Furthermore, the mechanisms involved are those that are broadly utilized across phylogeny for suppressing protein biosynthesis under conditions of energy (ATP) or substrate (amino acids) limitation such as occurs during starvation, hypoxia or other stresses (8,26). Key to translational suppression is reversible phosphorylation control over the activities of ribosomal initiation and elongation factors, studies to date having documented hibernation-responsive inhibition of the eukaryotic initiation factor 2 (eIF2) and eukaryotic elongation factor 2 (eEF2).

eIF2 introduces initiator methionyl-tRNA into the 40S ribosomal subunit. Phosphorylation of the alpha-subunit of eIF2 (eIF2α) inhibits this function because phospho-eIF2α acts as a dominant inhibitor of the guanine nucleotide exchange factor eIF2B and prevents the recycling of eIF2α between successive rounds of peptide synthesis (Figure 5) (8). Analysis of eIF2α again involves the use of dual antibodies detecting total eIF2α protein and phospho-eIF2α, the inactive form. As Figure3 shows, phospho-eIF2α content is markedly higher in kidney of hibernating, versus euthermic, ground squirrels with no difference in total eIF2α protein (17). Similar results were found in brain; phospho-eIF2α content in euthermic squirrels was <2% of the total but rose to ~13% during hibernation (11). Our newest studies have shown the same response during hibernation in multiple tissues of bats, *M. lucifugus*.

Although not yet assessed in hibernators, control over other initiation factors also contributes to the suppression of translation in situations such as starvation and hypoxia and these will likely prove to be involved in hibernation as well. For example, the eukaryotic initiation factor 5 (eIF5), which acts as a GTPase-activating protein to promote GTP hydrolysis within the 40S initiation complex (consisting of 40S*eIF3*AUG*Met-tRNA(f)*eIF2*GTP), is also regulated by reversible protein phosphorylation as is eukaryotic initiation factor 4E binding protein (4E-BP1) which, when dephosphorylated, binds to and inhibits eIF4E. Another subunit of eIF4, eIF4G, shows proteolytic fragmentation under stress (e.g. ischemia) and fragmentation changes the type of mRNA that can be translated because intact eIF4G is needed to allow eIF4E-bound m^7G-capped mRNAs (the vast majority of cellular mRNAs) to bind to the 40S ribosomal subunit (8). Without intact eIF4G, message selection changes dramatically to favour only those messages that contain an internal ribosome entry site (IRES) (12). In mammals, many messages that are translated using an IRES code for proteins involved in apoptosis but, significantly, the translation of several stress-responsive proteins is permitted by this mechanism under cellular conditions (e.g. hypoxia, amino acid limitation) that normally inhibit protein synthesis. For example, the mRNA of HIF-1α contains an IRES that allows enhanced synthesis of HIF-1α to occur under hypoxic conditions when overall protein synthesis is suppressed (19). The hiberna-

tion-responsive up-regulation of HIF-1α described earlier would undoubtedly occur via this mechanism.

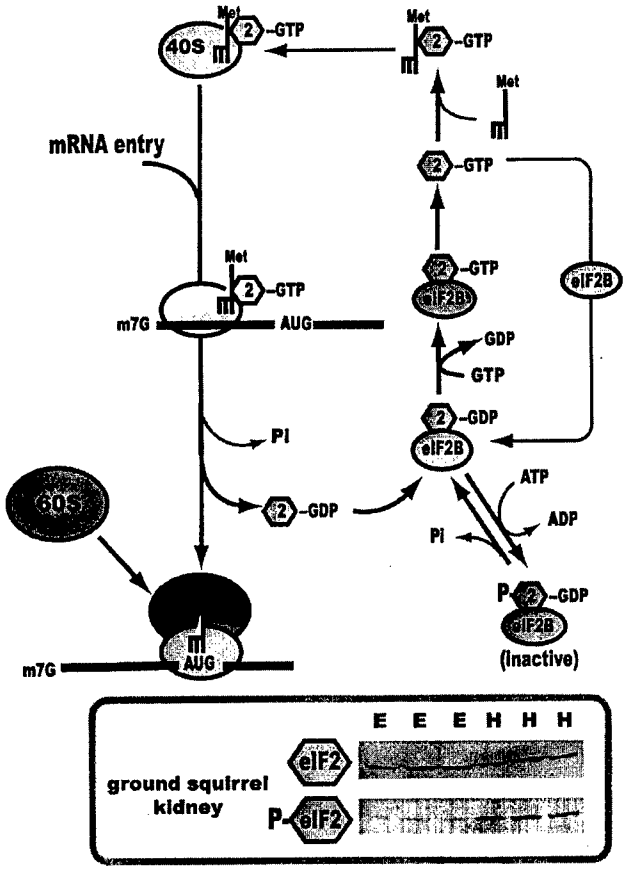

Figure 5. Role of the eukaryotic initiation factor 2 (eIF2) in transcription initiation. eIF2delivers the initiating methionine tRNA to the 40S ribosomal subunit. Phosphorylation of the alpha subunit of eIF2 inhibits translation because phospho-eIF2α acts as a dominant inhibitor of the guanine nucleotide exchange factor, eIF2B, and prevents the recycling of eIF2α between successive rounds of peptide synthesis. Inset shows the levels of total eIF2α protein and phospho-eIF2α content, as determined by Western blots, in three different kidney samples from euthermic (E) and hibernating (H) *S. tridecemlineatus* (17).

It appears, then, that hibernators make use of pre-existing mammalian mechanisms to inhibit protein synthesis when animals sink into torpor. The signal transduction cascade mediating this inhibition remains to be determined but it is hard to imagine how, for example, an amino acid limitation signal could be responsible within the short time frame of entry into torpor. However, as discussed above, a hypoxia signal might be contrived, the hypoxia-hypothermia connection engineering not only the overall drop in Tb but also inhibition of specific metabolic functions. Modified controls or an added layer of control

could sustain the inhibition in torpor even though the animal is not really hypoxic. Furthermore, it is seems probable that message selection (via an IRES or other mechanism) is the key to the synthesis of various proteins that have been identified as hibernation-responsive (discussed under transcriptional control). The likelihood that a hypoxia signaling pathway exerts control over protein synthesis (and/or other metabolic functions that are suppressed in hibernation) is of great current interest in our lab and is being explored by tracing the responses to hibernation of multiple elements (protein kinases, phosphatases, transcription factors) of the known mammalian signal transduction pathways involved in protein synthesis, FABP up-regulation, HIF signaling and others.

Protein translation is also regulated at the level of polypeptide elongation and reversible phosphorylation of elongation factors is again the mechanism. Frerichs *et al.* (11) demonstrated that mean transit times for polypeptide elongation by ribosomes were 3 times longer in extracts from brain of hibernating, compared with euthermic, ground squirrels. Subsequently, elevated amounts of phospho-eEF-2 were found in brain and liver of hibernating animals. Regulation was due to both an ~50% higher activity of eEF-2 kinase in hibernator tissues and a 20-30% decrease in protein phosphatase-2A activity (that opposes eEF-2 kinase) caused by a 50-60% increase in the levels of I_2^{PP2A}, the specific inhibitor of PP2A (7).

Ribosome Aggregation State

Another key factor in the regulation of protein synthesis is the state of ribosomal assembly. Active translation occurs on polysomes (aggregates of ribosomes moving along a strand of mRNA) whereas monosomes are translationally silent. Hence, an effective way to gauge the effects of a signal or stress on cellular protein synthesis activity is to analyze its effects on the distribution of ribosomes between polysome and monosome fractions. Several stresses that are known to compromise cellular energy or amino acid availability (e.g. hypoxia, starvation, diabetes) cause polysome disaggregation. Disaggregation also occurs in natural states of hypometabolism (e.g. anoxia tolerant organisms) (26).

The state of polysome assembly is assessed by separating ribosomes on a sucrose gradient; polysomes appear in the denser fractions and monosomes and messenger ribonuclear proteins are in the lighter fractions. Northern blotting is used to detect individual mRNA transcript types within the gradient whereas ribosome presence can be quantified by detecting rRNA via absorbance at 254 nm, ethidium bromide staining, or Northern blotting with a ^{32}P-labelled 18S rRNA probe. Recent studies using this technique with several tissues (kidney, liver, brain) of ground squirrels have consistently shown a disaggregation of polysomes during hibernation and a shift of mRNA for constitutively-active genes into the monosome fraction (11,17,18). For example, studies with kidney from *S. tridecemlineatus* showed a bimodal distribution of the mRNA for a constitutively expressed gene, cytochrome c oxidase subunit 4 (*Cox4*), between the heavy polyribosome fractions and the monosome/mRNP fractions in extracts from euthermic animals. However, when hibernator kidney was assessed, *Cox4* mRNA transcripts were strongly shifted into the monosome fraction indicating that they were not being translated during torpor (17). A comparable result was seen for *Cox4* in BAT (Figure 6) and for another constitutive gene (glyceraldehyde-3-phosphate dehydrogenase) in liver (18). Furthermore, a temperature dependence of polysome disaggregation in liver was demonstrated. Using samples from ground squirrels

sacrificed at different Tb values during entry into and arousal from hibernation, Van Breukelen and Martin (28) showed that the distribution of ribosomal RNA and actin mRNA (a constitutively active gene) showed a marked shift to the monosome fraction when body temperature fell to 18°C and below. Similarly, reaggregation of polysomes occurred when this Tb was exceeded during arousal. Whether this effect of temperature on ribosome aggregation state is a passive influence of temperature or results from reversible regulation of one or more of the initiation factors remains to be seen.

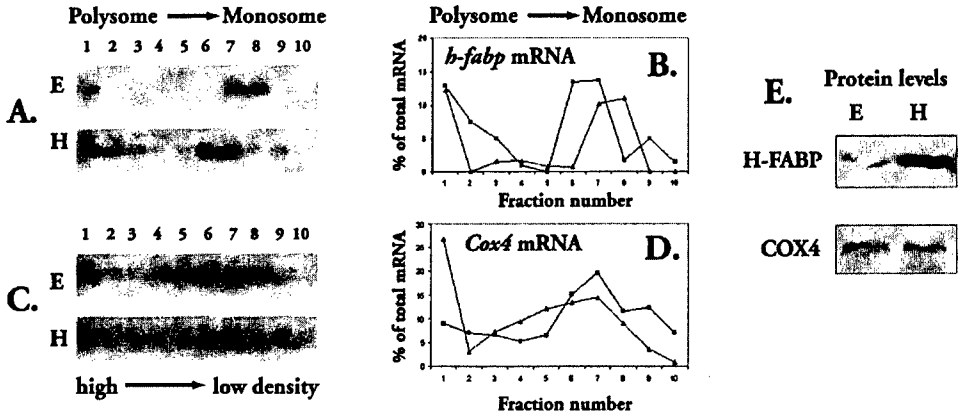

Figure 6. Polysome profiles of brown adipose tissue from euthermic (E) and hibernating (H) ground squirrels, *S. tridecemlineatus*. Ribosomes were separated on a sucrose gradient and then drained in 10 fractions. Northern blots tracked *h-fabp* (A) or *Cox4* (B) transcript levels in each fraction and the graphs on the right (B,D) show the percentage of total transcript content in each fraction (triangles show euthermic values, squares show hibernating). Polysomes are in the high density fractions (low fraction numbers). Effects of hibernation on H-FABP and COX4 protein levels, as assessed by Western blots, are shown in E. Data compiled from (17).

Hence, polysome disaggregation appears to be one of the key features of translational inhibition during hibernation. However, if mRNA transcripts are generally sequestered into the monosome fraction during hibernation, how do we account for the multiple instances of hibernation-responsive gene up-regulation that were discussed above? Our recent studies with *S. tridecemlineatus* are highlighting some interesting variations on the general principle.

The first of these is differential distribution of individual mRNA species between polysome and monosome fractions. Not all polysomes disappear during hibernation and those that remain can continue translation of selected messages. Figure 6 shows the example of ground squirrel BAT. As discussed earlier, fatty acid binding proteins are up-regulated in BAT during hibernation. When we looked at the ribosomal distribution of the up-regulated *h-fabp* transcripts compared with the transcripts of the constitutively expressed gene *Cox4* in extracts from euthermic versus hibernating squirrels, distinct differences were seen (17). A high proportion of total *h-fabp* message was associated with the heaviest polysomes in both states (Figure 4b) and combined with the strong increase in total *h-fabp* message that is illustrated by the Northern blots (Figure 4a), this represented a substantial enrichment

of *h-fabp* transcripts in the polysome fractions from hibernating animals. By contrast, total *Cox4* message did not change during hibernation (Figure 4c) but transcripts were largely sequestered into the monosome fractions (Figure 4d). As predicted from this ribosomal distribution, H-FABP protein content increased strongly in hibernator BAT but COX4 protein did not change (Figure 4e). This shows that individual transcript species can be treated very differently during hibernation and identifies the differential distribution of individual mRNA species between translationally active and inactive ribosomes as a principle of metabolic regulation in hibernation. The heavy polysomes in hibernator BAT contain (and presumably translate) those mRNAs (such as *h-fabp*) that are crucial to the hibernation phenotype whereas mRNA species that are not needed during hibernation are relegated into the translationally silent monosome fractions.

Another variation on translational control in hibernation is the concept of anticipatory up-regulation of genes. Transcript levels of some genes are elevated during hibernation but no increase in the corresponding protein product occurs. We first encountered this paradox while studying the gene for the organic cation transporter type 2 (*Oct2*) in *S. tridecemlineatus* kidney (17).Organic cation transporters are transmembrane protein pumps that actively absorb and/or excrete endogenous and exogenous organic ions against their concentration gradients (27). OCT2 protein is found primarily in kidney where it is localized in the basolateral membranes of cells lining the proximal tube (outer medulla) of the nephron. *Oct2* was first identified as hibernation-responsive from cDNA array screening and subsequent Northern blotting confirmed an approximate two-fold up-regulation. Nonetheless, OCT2 protein decreased by 66% in hibernating, versus euthermic, kidney (17). The explanation for this dichotomy came from the analysis of *Oct2* transcript distribution on the ribosome profiles. These showed that although *Oct2* transcripts levels were much higher in hibernator extracts, virtually all of the mRNA transcripts were sequestered into the monosome fraction. In the euthermic state, by contrast, *Oct2* transcripts were distributed approximately equally between polysome and monosome fractions. Hence, *Oct2* was up-regulated in hibernation but not translated. Why would this odd behaviour occur? One reason may be that OCT2 protein could be particularly sensitive to some form of damage that accrues during hibernation; for example, the protein may be damaged by oxygen free radicals or by low temperature, leading to its degradation. Another possibility is that part of the process of shutting down kidney function during hibernation may be the suppression of membrane transport functions that are major energy consumers. Some transporters may be controlled with reversible mechanisms such as protein phosphorylation whereas deactivation of others may be only possible via protein degradation. In either case, it could make sense that the *Oct2* gene is up-regulated as animals enter hibernation and its transcripts stored in the monosome/mRNP fractions during torpor. That way the transcripts are present and ready to be translated as soon as possible when arousal begins in order to allow the fastest possible restoration of OCT2 protein levels to support the resumption of kidney functions during the brief hours of the interbout.

Hence, multiple mechanisms of translational control are available to the hibernator and these are used to accomplish a variety of specific goals including a general suppression of protein translation (via inhibition of transcription factors and mRNA sequestering into the monosome fraction), the specific up-regulation of selected transcripts (IRES-mediated translation, preferential transcript presence in polysomes), and anticipatory up-regulation but delayed translation of other transcripts (transcript up-regulation but sequestered into

the monosome fraction). All these mechanisms combined contribute the regulated suppression of the protein synthesis as part of the general metabolic rate depression during hibernation while still providing the means to continue to produce selected proteins that are key to the hibernation phenotype.

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Chapter 4

OXYGEN CONFORMANCE OF CELLULAR RESPIRATION

A perspective of mitochondrial physiology

Erich Gnaiger

Abstract: Oxygen pressure declines from normoxic air-level to the microenvironment of mitochondria where cytochrome *c* oxidase (COX) reduces oxygen to water at oxygen levels as low as 0.3 kPa (2 Torr; 3 μM; 1.5 % air saturation). Intracellular hypoxia is defined as (1) local oxygen pressure below normoxic reference states, or (2) limitation of mitochondrial respiration by oxygen levels below kinetic saturation, resulting in oxyconformance. High-resolution respirometry provides the methodology to measure mitochondrial and cellular oxygen kinetics in the relevant low oxygen range <1 kPa (7.5 mmHg; 9-10 μM; 5 % air saturation). Respiration of isolated heart mitochondria follows hyperbolic oxygen kinetics with half-saturating oxygen pressure, p_{50}, of 0.04 kPa (0.3 Torr; 0.4 μM) in ADP-stimulated state 3. Thus mitochondrial respiration proceeds at 90 % of its hyperbolic maximum at the p_{50} of myoglobin, suggesting the possibility of a small but significant oxygen limitation even under normoxia in active muscle. Any impairment of oxygen delivery, therefore, induces oxyconformance. In addition, a shift of mitochondrial oxygen kinetics to the right, particularly by competitive inhibition of COX by NO, causes a further depression of respiration and a compensatory increase of local oxygen pressure. Above 1 kPa, mitochondrial oxygen uptake increases above hyperbolic saturation, which is probably due to oxygen radical production rather than the kinetics of COX. In cultured cells, the pronounced oxygen uptake above mitochondrial saturation at air-level oxygen pressure cannot be inhibited by rotenone and antimycin A, amounting to >20 % of routine respiration in fibroblasts. Biochemical models of oxyconformance of COX are evaluated relative to patterns of intracellular oxygen distribution in the tissue and enzyme turnover *in vivo*, considering the kinetic effects of COX excess capacity on flux through the mitochondrial electron transport chain.

Key Words: oxygen kinetics, cytochrome *c* oxidase, mitochondrial respiratory control, oxygen limitation, hypoxia

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
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INTRODUCTION

The high affinity of cytochrome *c* oxidase for oxygen implies independence of mitochondrial respiration of oxygen over a wide range of oxygen levels, which gives rise to the paradigm of "oxygen regulation", although "kinetic oxygen saturation" describes more accurately the underlying mechanism. In contrast, various degrees of oxyconformance are observed in cells (2, 9, 28, 33, 36). Biochemical and physiological approaches are required to separate the primary kinetic mechanisms from secondary effects of oxygen sensing, signalling, gene expression and protein synthesis or degradation. Modern trends in mitochondrial bioenergetics integrate *(1)* molecular and enzyme kinetic properties of the membrane proteins constituting the electron transport chain, particularly the proton pumps such as cytochrome *c* oxidase (70), *(2)* synkinetic properties of the mitochondrial metabolic network involved in the control of flux and energetic efficiency (26, 27), and *(3)* the regulatory role of mitochondrial signalling in the cell and of intracellular conditions in the tissue. From such studies concepts emerged on reactive oxygen species (ROS) signalling cascades (37, 44), redox signalling (34), the protective role of regulated low intracellular pO_2 (22, 27, 58), and the mitochondria-dependent pathway of controlled cell death or apoptosis (4).

Approaching the problem of hypoxia and oxygen dependence of respiration from such a perspective of mitochondrial physiology, this review *(1)* relates classical enzyme kinetics of cytochrome c oxidase with mitochondrial respiratory control, *(2)* contrasts mitochondrial oxygen kinetics and oxygen dependence of cellular respiration, *(3)* illustrates the importance of oxygen diffusion in determining oxygen conformance of respiration in various cell types and tissue preparations, and *(4)* discusses concepts on the energetics of metabolic downregulation under hypoxia in the light of these baseline studies.

HYPOXIA OR HYPEROXIA IN ISOLATED AND CULTURED CELLS

Several apparent paradoxes have emerged in the physiology and pathology of hypoxia, such as the oxygen, lactate, efficiency, and diving paradoxes (32). While some have been rationalized and solved, others remain hot spots of current research. Another apparent paradox on hypoxia arises in studies of the bioenergetics of isolated and cultured cells, where respiration, contractile performance or protein synthesis are apparently oxygen limited at partial pressures at or above normoxic tissue levels. Such extended oxygen conformance deviates from the "regulatory" pattern or oxygen independence of mitochondrial respiration to <1 kPa (7.5 mmHg (28)). Respiration of various chronically or acutely exposed cell types is partially oxygen dependent up to >50 % air saturation (2, 33, 54, 61). The response pattern is biphasic and corresponds to microxic regulation, characterized by a steep increase of flux at low oxygen and a more shallow oxyconformance at high oxygen levels (21).

Compared with ambient oxygen pressure of 20 kPa (150 mmHg), oxygen levels are low within active tissues and are under tight control by microcirculatory adjustments to match oxygen supply and demand. Alveolar normoxia of 13 kPa (100 mmHg) contrasts

with a corresponding 1 to 5 kPa (10 to 40 mmHg) extracellular pO_2 in solid organs such as heart, brain, kidney and liver (19). Considering the respiratory cascade and oxygen in the microenvironment of tissue (23, 26), it appears surprising that protein synthesis becomes inhibited in hepatocytes incubated at a "hypoxic" pO_2 of 11 kPa (80 mmHg) compared with 95 % oxygen (41), hepatocyte respiration is reduced at 9 kPa (70 mmHg (54)), and cytochrome *c* oxidase is reversibly inhibited at 50 μM (4 kPa or 30 mmHg (16)). Does this suggest substantial oxygen limitation of aerobic ATP production and protein synthesis to prevail under normoxia *in vivo*, or are responses to oxygen altered *in vitro*?

Protein synthesis in isolated cardiomyocytes is inhibited at 0.05 kPa (0.4 mmHg) but not at 0.5 kPa. Casey *et al.* (12), therefore, suggest that part of the apparent oxygen paradox may be due to oxygen gradients giving rise to differences between the gas phase and the cell level. Pericellular pO_2 falls to <0.03 kPa (0.2 mmHg) in human hepatoma cells growing in monolayer culture with 95 % air in the gas phase, when respiration is significantly oxygen limited (43). Continuous cultures of mouse hybridoma cells grow with optimum yield at 0.5 % air saturation (0.5 kPa; 4 mmHg; (45)), and the biochemical efficiency of ATP production per oxygen consumed is high in isolated mitochondria under severely limiting hypoxia at pO_2 as low as 0.002 kPa (0.014 mmHg; (27)). Advancements in the study of mitochondrial and cellular oxygen kinetics may help to clarify some controversial aspects of respiratory control under hypoxia.

HIGH-RESOLUTION RESPIROMETRY AT LOW LEVELS OF OXYGEN

Our studies on the oxygen dependence of mitochondrial (Figs. 1 and 2), cellular (Figs. 3 and 4) and tissue respiration (Figure 6) are based on high-resolution respirometry with the twin-chamber OROBOROS® *Oxygraph* with chamber volumes set at 2 ml. The software *DatLab* (OROBOROS, Innsbruck, Austria) is used for data acquisition (1 or 2 s time intervals) and analysis, including calculation of the time derivative of oxygen concentration to obtain a continuous on-line record of flux, signal deconvolution dependent on the response time of the oxygen sensor, and oxygen-dependent correction for instrumental and chemical background oxygen flux. Oxygen back-diffusion at low oxygen is minimized in this system by the use of gas-impermeable materials, with glass chambers, titanium stoppers, PEEK-coated magnetic stirrer bars, viton O-rings and butyl rubber sealings. High signal stability and dynamic background correction for oxygen consumption of the oxygen sensor and oxygen backdiffusion provide the basis for high resolution of oxygen flux (<2 $pmol.s^{-1}.cm^{-3}$). Signal noise decreases with decreasing oxygen to less than ±0.003 kPa (0.02 mmHg; recorded near zero oxygen over 100 data points and 1 s intervals), which is of particular advantage for studies in the range of physiological intracellular oxygen levels and hypoxia. A dynamic correction for sensor drift at zero oxygen pressure extends the sensitivity to this low noise level. Instrumental design, experimental procedures and data analysis are described in detail elsewhere (22, 28, 61).

EXCESS CAPACITY OF CYTOCHROME *C* OXIDASE AND RESPIRATORY FLUX CONTROL

The terminal acceptor for oxygen of the mitochondrial electron transport chain, cytochrome *c* oxidase (COX), has the capacity to operate at a turnover rate of 300 electrons per second when studied as an isolated enzyme (49). When the enzyme remains integrated in the inner membrane of isolated mitochondria, however, the turnover rate is about 6-fold less. This is the case when complex IV of the respiratory chain (COX) is studied as an isolated step, by blocking complex III with antimycin A and feeding electrons from the artificial substrate TMPD to cytochrome *c* and COX (Figure 1). Under these conditions, electron flux is coupled to proton translocation and is stimulated by ADP. The reaction velocity depends further on the concentration of reduced TMPD (500 μM in our studies), which is held constant by an excess concentration of ascorbate (2 mM; Figure 1). In heart mitochondria, even this COX turnover rate is twice above the maximum physiological value, which is measured as oxygen flux through the entire respiratory chain with the physiological substrates pyruvate and malate (Figure 1).

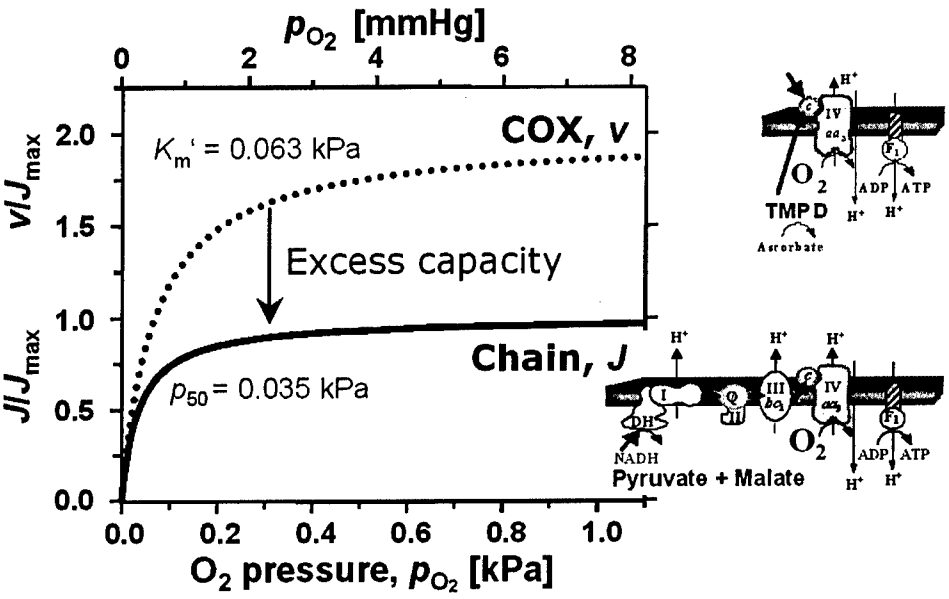

Figure 1. Oxygen kinetics in isolated heart mitochondria for reaction velocity, *v*, of the isolated step of complex IV (cytochrome *c* oxidase, COX (23)) and for oxygen flux, *J*, through the respiratory chain (25). Coupled respiration was stimulated to state 3 by ADP. Maximum reaction velocity of COX with 0.5 mM TMPD, 2 mM ascorbate and 2.5 μM antimycin A was about two times higher than respiratory flux with 5 mM pyruvate and 2 mM malate. Proportional to COX turnover, the apparent half-saturation constant, K_m', was about two times higher for COX (0.47 mmHg) than the p_{50} for the respiratory chain (0.26 mmHg). The oxygen range for kinetic analysis corresponds to 10 μM O_2 or 5 % air saturation.

The oxygen kinetics of cytochrome *c* oxidase in mitochondria follows a monophasic hyperbolic function over an oxygen concentration range >10 times the apparent half-saturation or Michaelis-Menten constant, K_m'. The K_m' of COX in rat heart mitochondria is 0.67 ± 0.12 μM (23), equivalent to 0.063 kPa or 0.47 mmHg (Figure 1). The K_m' is not a constant but depends on enzyme turnover (69), hence the physiological p_{50} of mitochondrial respiration is even lower at 0.035 kPa or 0.26 mmHg at maximum activity (state 3; Figure 1). The p_{50} is further attenuated at submaximal activity, dropping off to 0.014 kPa or 0.1 mmHg in the resting state of ADP-limited respiration (26). These values illustrate the specific demand imposed on high-resolution respirometry for accurately measuring and maintaining low levels of dissolved oxygen in suspensions of isolated mitochondria and cells (22).

Biochemical determination of the kinetics of cytochrome *c* oxidase is insufficient to predict the oxygen dependence of mitochondrial or cellular respiration. A synkinetic systems approach is required to explain tissue-specific differences in mitochondrial oxygen affinity, which is a function of the properties of the electron transport pathway (25, 26). The excess capacity of COX ensures that this enzyme operates far from its limiting turnover capacity even at maximum activity of the respiratory chain. When the excess capacity of COX is reduced, then COX is pushed to increasing turnover at identical rates of mitochondrial respiration. As a consequence, the mitochondrial p_{50} declines. Downregulation of cytochrome *c* oxidase activity, therefore, increases the degree of oxyconformance in the low-oxygen range (Figure 1). Reversible inhibition of COX by nanomolar levels of NO induces oxyconformance to a much higher extent (8). An entirely different mechanism for the control of oxyconformance has been proposed by Chandel *et al.* (16), based on reversible downregulation of isolated COX after conditioning at oxygen levels of 15-30 mmHg (2-4 kPa). This putative control of mitochondrial respiration by allosteric changes of COX is unconvincing for two reasons. *(1)* Some hours of conditioning is required for the isolated enzyme, whereas the oxygen effect is instantaneous on embryonic cardiomyocytes (9), and *(2)* owing to the high excess capacity of this enzyme (100 % according to Figure 1; or even 400 % according to data of Budinger *et al.*, (9)), over-proportional inhibition of COX is required but was not found to explain downregulation of cellular respiration. In the heart and to a lesser extent in the liver, pathway flux is limited at kinetic oxygen saturation by electron input into COX from the respiratory chain, as expressed by the excess capacity of COX and reflected in a low flux control coefficient (25, 26). Cytochrome *c* oxidase exerts increasing control over respiration at severely limiting oxygen levels <0.1 kPa or <1 mmHg (23).

We were concerned about the potential effect of the time course of oxygen depletion in the closed-chamber respirometer on the respiratory capacity and oxygen kinetics of isolated mitochondria. Changes of mitochondrial protein concentration influence the aerobic-anoxic transition time at any given metabolic state. Using various dilutions of rat liver mitochondria, the transition time between kinetic oxygen saturation at 1.1 kPa (10 mmHg) and anoxia was varied 10-fold in the range of 30 to 300 s, which did not exert any influence on the mitochonrial p_{50} (40). Such kinetic independence was also reported for isolated heart mitochondria (25). Similarly, repeated aerobic-anoxic transitions with intermittent reoxygenations to low oxygen levels did not result in an increase of the p_{50}, when mitochondria were maintained for 2 h at oxygen pressures <2.5 kPa (<20 mmHg). On the contrary, the p_{50} declined moderately as a function of the time-dependent loss of respiratory capacity (Figure 2). Respiratory instability was obtained with time of exposure, even when

mitochondria were maintained continuously above 8 kPa (60 mmHg) oxygen pressure (30 °C), ruling out the possibility that low oxygen or repeated ischemia-reperfusion were responsible for mitochondrial injury (Figure 2). The main mitochondrial defects of long-term exposure were *(1)* cytochrome *c* release (reversed by addition of external cytochrome *c*; Figure 2) which results in increased mitochondrial superoxide production (13), and *(2)* limitation of the phosphorylation system (reversed by uncoupling with FCCP; Figure 2; see also ref. (3)). Mitochondrial respiration is more stable in an improved mitochondrial medium (24). In agreement with a study by Taylor *et al.* (65), these results do not support the hypothesis that oxyconformance of mitochondrial respiration is caused by conditioning of cytochrome *c* oxidase during exposure to oxygen levels of 2 to 20 mmHg or even up to 50 μM (4 kPa or 30 mmHg (15, 16)).

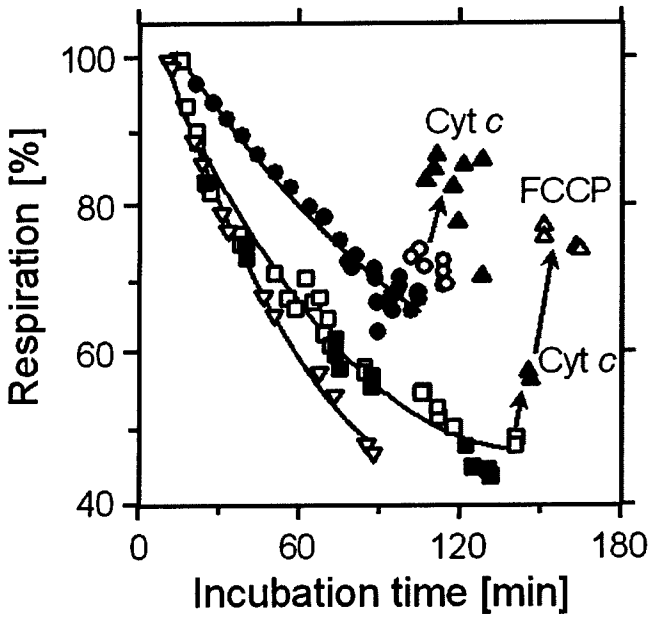

Figure 2. Decline of respiration in isolated rat liver mitochondria as a function of time during incubation in various oxygen regimes: always >80 μM (8 kPa or 60 mmHg; open downward triangles); series of aerobic-anoxic transitions with reoxygenations to high oxygen levels (>25 μM; 2.5 kPa or 20 mmHg; open squares and open circles), or aerobic-anoxic transitions with maximum oxygen levels always maintained <25 μM (closed symbols). Addition of catalase to the medium improved respiratory stability (circles). Oxygen flux is expressed relative to initial respiratory rates. Non-linear fits indicate trends in experiments with identical conditions. Addition of 10 μM cytochrome *c* partly restored respiration. A further increase was obtained after uncoupling by 2 μM FCCP (arrows). The incubation medium MiR03 contained 10 mM succinate, 0.5 μM rotenone, 1 mM ATP, >1 mM ADP, 200 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, 1 g/l BSA, 3 mM $MgCl_2$, 20 mM taurine and 10 mM KH_2PO_4 (pH 7.1; 30 °C; from Lassnig *et al.*, (40)).

BIPHASIC OXYGEN KINETICS: MITOCHONDRIAL AND NON-COX RESPIRATION

Hyperbolic Michaelis-Menten kinetics suggests substrate saturation of flux, theoretically reaching 98 % of maximum at a substrate concentration of 50 times the p_{50} (corresponding to 2 kPa or 15 mmHg at a p_{50} of 0.04 kPa). In many cases, hower, mitochondrial and cellular respiration continues to increase significantly at such high oxygen levels, resulting in biphasic oxygen kinetics (28). Oxygen dependence of respiration in this high-oxygen range up to air saturation has escaped detection in many cases, as illustrated by an experimental example with fibroblasts (33). A continuous decline of oxygen concentration is obtained over time in a closed chamber respirometer (Figure 3A; cO_2). This decline might be approximated by a straight line on a conventional chart recorder trace, which then would imply a constant respiratory flux (the negative slope) and oxygen-independence to very low oxygen levels. Continuous calculation of the time derivative of digitally recorded oxygen concentration, however, clearly reveals an oxygen-dependent attenuation of respiration even at >2 kPa or 20 µM (Figure 3A; JO_2). Cellular oxygen kinetics is hyperbolic when zooming into the low-oxygen range <1 kPa or 10 µM (Figure 3B). A kinetic plot over the full oxygen range illustrates the biphasic oxygen dependence of respiration in human fibroblasts and endothelial cells (Figure 4). This biphasic pattern is not restricted to cells but is observed in isolated mitochondria (28).

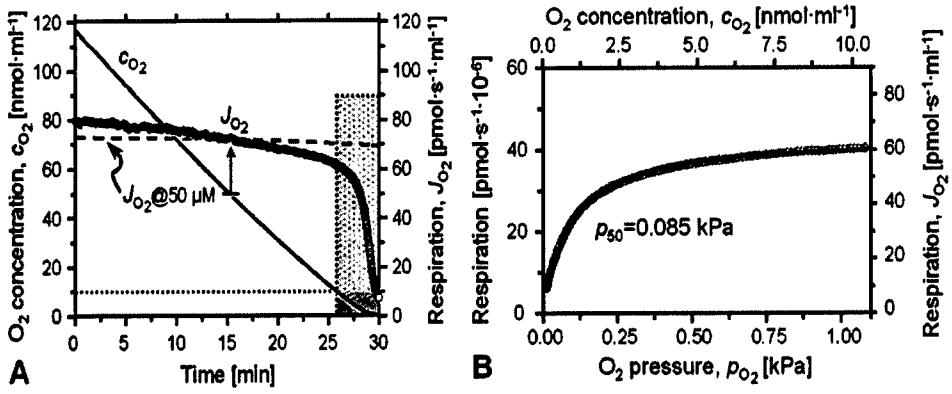

Figure 3. High-resolution respirometry with suspended human foreskin fibroblasts in the Oroboros *Oxygraph-2k*. **A.** Oxygen concentration, cO_2, and respiratory oxygen flux, JO_2, as a function of time in an aerobic-anoxic transition. The significant decline of oxygen flux at high oxygen (circles) was oxygen dependent, whereas loss of respiratory capacity with time (stippled line) contributed to only a small extent. The stippled line shows interpolations of oxygen flux, JO_2, at 50 µM O_2 measured in repeated aerobic-anoxic transitions before and after this section of the experiment. The shaded square indicates the section of the low oxygen range (dashed: concentration; dotted: flux), over a time interval of 3.8 min or 227 s. **B.** Kinetic plot of respiration as a function of oxygen concentration or partial pressure in the low oxygen range. Data points are shown by circles, where flux is calculated at 2-s time intervals with corrections for the exponential response time of the oxygen sensor and the oxygen dependence of instrumental background oxygen flux. Maximum respiration was calculated at 43.4 pmol·s^{-1}·10^{-6} cells, and the oxygen pressure at half-maximum respiration, p_{50} was 0.089 kPa (0.67 mmHg, calculated in the 1.1 kPa range). Modified after Hütter *et al.* (33).

The oxyconforming component of respiration was calculated as the difference between total cellular respiration (Figure 4A; upper trace) and the hyperbolic component in the low-oxygen range (Figure 3B; extrapolated in Figure 4A; dotted line). This oxyconforming component was proportional to oxygen pressure, although a saturation effect towards air saturation cannot be excluded (Figure 4). After uncoupling by FCCP, respiration was inhibited by rotenone and antimycin A in independent experiments at various levels of oxygen (33). Rotenone and antimycin-A are effective inhibitors of complexes I and III of the respiratory chain and thus inhibit electron transport to cytochrome *c* oxidase. Similar to the oxyconforming component of respiration, the inhibited oxygen uptake of cells (non-COX respiration) increases proportional to oxygen pressure in the experimental range (Figure 4A; circles).

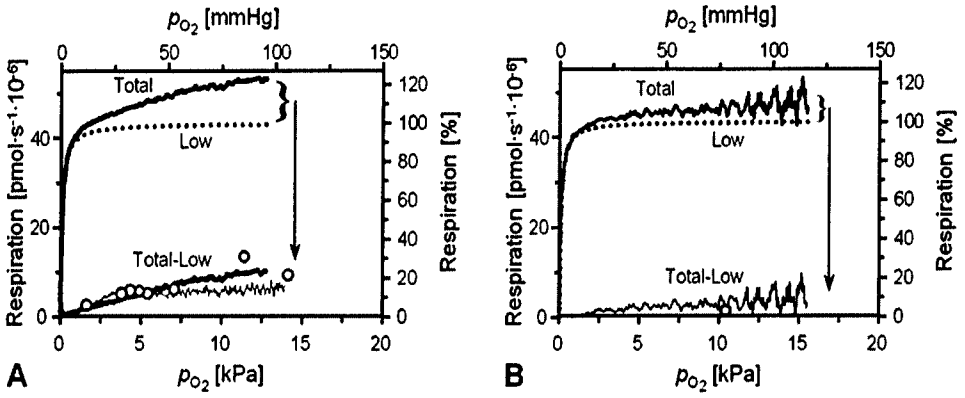

Figure 4. Oxygen dependence of routine respiration (pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells) in culture medium of (**A**) human fibroblasts ($1.5 \cdot 10^6$ cells per ml; from Figure 3; Dulbecco's modified Eagles's medium) and (**B**) human umbilical vein endothelial cells (HUVEC; $0.8 \cdot 10^6$ cells per ml; in EGM). Oxygen kinetics is biphasic over the full experimental oxygen range. The dotted line shows the extrapolation of the monophasic hyperbolic relation calculated over the standard low oxygen range (<1.1 kPa) for analysis of mitochondrial oxygen kinetics (Low; A: from Figure 3B; B: maximum respiration of HUVEC was 43.7 pmol·s^{-1}·10^{-6} cells or 34 pmol·s^{-1}·ml^{-1}; p_{50}=0.074 kPa or 0.56 mmHg). At high oxygen, the difference between total cellular respiration (Total) and the extrapolated hyperbolic fit was directly proportional to oxygen pressure (Total-Low; A: solid traces show the difference calculated from two aerobic-anoxic transitions). Open circles show respiration inhibited by rotenone and antimycin A. Modified after Hütter *et al.* (33) (A) and Gnaiger *et al.* (28) (B).

80-90 % of the oxygen consumed by an organism is considered to be reduced to water by cytochrome *c* oxidase, the terminal enzyme of the respiratory chain (34, 49, 50). Appart from COX, therefore, a significant potential (10-20 % of total respiration) exists for oxygen utilization by the >100 known oxidoreductases with dioxygen as substrate and by autoxidation of reduced compounds in the cell. Although it is well established that many oxidases, such as xanthine oxidase or monoamino oxidase, have K_m' values for oxygen more than two orders of magnitude higher than COX (68), surprisingly little attention has be paid to the oxygen dependence of non-COX respiration or non-mitochondrial respiration in intact

cells (58). When measured close to air saturation, the non-mitochondrial contribution to organismic respiration is overestimated significantly, owing to the low intracellular oxygen levels in tissues and the strong oxyconformance of non-COX respiration (Figure 4). The conventionally assumed 10 % share to total respiration needs downward correction even under normoxia. Although a quantitatively minor component of total respiration, hypoxic limitation of non-COX oxygen consumption has potentially important consequences on redox signalling (37, 44), biosynthetic reactions with a requirement of molecular oxygen (66), and perhaps on oxidative repair of damaged DNA and RNA (1).

In beef heart submitochondrial particles, hydrogen peroxide and superoxide radical production increase near-linearly with oxygen pressure from 0 to 100 kPa (pure oxygen saturation (6)). ROS production is reduced under hypoxia in pulmonary but not renal artery mitochondria (44). NADH-ubiquinone reductase (complex I) and ubiquinol-cytochrome *c* reductase (complex III) comprise the main sites of electron leak, although in various cell types mitochondrial glycerophosphate dehydrogenase-dependent hydrogen peroxide production represents another effective branch for the electron leak (18). Their common compound ubisemiquinone provides the electrons for mitochondrial non-COX oxygen consumption and ROS production (6),

$$J = pO_2 \cdot (UQH^{\bullet}) \cdot k \qquad (1)$$

The concentration of reduced intermediates potentially reacting with dioxygen, such as ubisemiquinone, ($UQH^{\bullet}$), depends on metabolic state (7) and mitochondrial type (44). In addition, nitric oxide not only inhibits complexes I and IV of the respiratory chain, but regulates mitochondrial production of H_2O_2 (7, 53). In general, therefore, mitochondrial superoxide radical and hydrogen peroxide production are not simple functions of pO_2, which renders reaction (1) an ambiguous or versatile oxygen sensor. Components of the electron transport chain become over-proportionally reduced under conditions of excessive substrate supply and low pO_2. Despite progressive oxygen limitation (Eq. 1), therefore, electron leak and ROS production may increase under hypoxia and reductive stress (17, 42).

In heart mitochondria, rotenone inhibits H_2O_2 production, but subsequent addition of antimycin A restores or even stimulates the rate of hydrogen peroxide generation (6). Importantly, rotenone-inhibited cellular oxygen consumption remains constant after addition of antimycin A (33), which may be taken as indirect evidence for a significant non-COX contribution to rotenone/antimycin A-inhibited respiration in fibroblasts. Even the mitochondrial outer membrane monoamino oxidase activity may surpass hydrogen peroxide production by the inner mitochondrial membrane (7). Consequently, respiration inhibited by antimycin A and particularly by cyanide cannot simply be interpreted as non-mitochondrial respiration. Caution is required since cyanide is not specific for cytochrome *c* oxidase but is a direct inhibitor of other oxidases, such as urate oxidase (56) and inhibits the heme-containing catalase (20). Cyanide inhibition can in fact depress cellular respiration close to zero, particularly after cell membrane permeabilization when the soluble reducing cytosolic components are released and diluted in the mitochondrial incubation medium (48).

A simple kinetic model may explain at least in part the biphasic pattern of respiration observed in small cells with minor intracellular oxygen gradients. The p_{50} measured in the well-mixed incubation medium of these cells is close to the p_{50} of isolated mitochondria

(Figure 1 and Figure 3B (61)). With a lumped apparent K_m' for all non-COX reactions that is 100 times higher than the p_{50} (10 versus 0.1 kPa), total cellular respiration can be simulated in the range of anoxia to air saturation (Figure 5A). Moreover, the oxyconforming non-COX component of respiration is shown to be insignificant in the low-oxygen range used for calculating the hyperbolic fit (Figure 5B). An extension of these studies up to pure oxygen saturation will help elucidating the contribution of non-saturable autoxidation processes to the oxyconformance of cellular respiration. On the other hand, oxygen kinetics of purified COX needs to be studied by high-resolution respirometry to test the hypothesis that the biphasic pattern of oxyconformance is exclusively due to mitochondrial and non-mitochondrial mechanisms of oxygen consumption which are not related to cytochrome *c* oxidase.

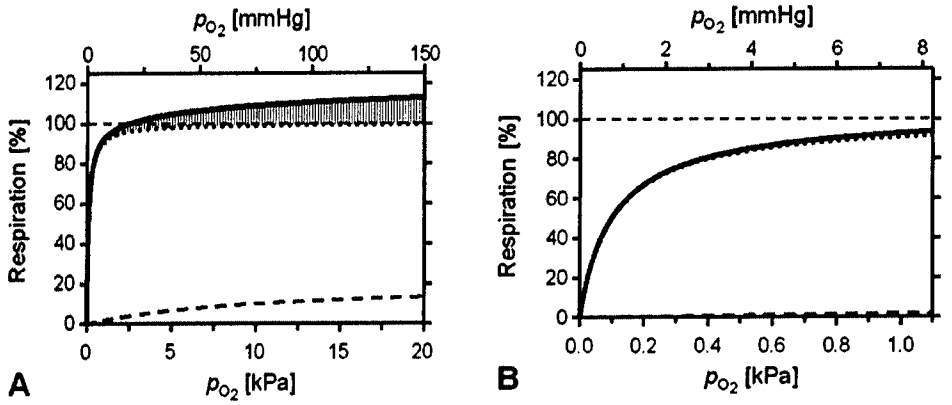

Figure 5. A simple model of biphasic oxygen kinetics in cells, including mitochondrial kinetics with an extracellular p_{50} of 0.1 kPa (0.75 mmHg; dotted lines; maximum oxygen flux at 100 %), and the kinetics of various oxidases with a lumped apparent K_m' of 10 kPa (75 mmHg; assuming these oxidases reach 20 % of mitochondrial respiration at kinetic saturation >100 kPa; dashed lines). Total respiration (full lines) is the sum of the mitochondrial and the oxyconforming non-COX components. **A**: Oxygen range up to air saturation, when the lumped oxidases reach 67 % of their maximum capacity. **B**: Low oxygen range of mitochondrial kinetics, showing that the error is negligable when calculating a hyperbolic fit for total respiration at pO_2<1.1 kPa.

OXYGEN DIFFUSION AND OXYCONFORMANCE

Suspended endothelial cells are spherical with a radius of 5-7 μm, but diffusion distances to mitochondria are reduced owing to the large nucleus which occupies a significant fraction of the central cellular volume (26). Correspondingly, intracellular oxygen gradients are small in endothelial cells (61) and fibroblasts (0.0013 and 0.0028 nL volume per cell, respectively (33)). Routine respiration of endothelial cells is 30 to 40 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells, and is increased 2.5- to 3.5-fold by uncoupling (59, 61). By comparison, rod-shaped adult cardiomyocytes are large (Table 1). Compared to routine respiration in endothelial cells and fibroblasts, activated or uncoupled cardiomyocytes respire at a 50- to

100-fold higher oxygen flow per cell, i.e. 2,000 to 4,000 pmol·s^{-1}·10^{-6} cells ((36, 47, 71) corrected to 37 °C with a Q_{10} of 1.8 (46)). Correspondingly, significant intracellular oxygen gradients (64) give rise to a 10-fold difference between the mitochondrial p_{50} (Figure 1) and extracellular p_{50} values determined in active cardiomyocytes (Figure 6).

Diffusion limitation is further aggravated in permeabilized fiber bundles with a radius of 35 up to 200 μm (38, 52). For comparison, 200 μm away from the nearest blood vessel, the pO_2 drops from 1.9 kPa (14 mmHg) to zero in tumors with relatively low aerobic capacity (30). In permeabilized myocardial fiber bundles the microcirculation is disrupted, myoglobin is released, and the mass-specific respiratory activity is 600 pmol·s^{-1}·mg^{-1} dry weight in the ADP-activated state 3 ((24) measurements converted from 30 °C to 37 °C with a Q_{10} of 1.8; compared to 2,000 pmol·s^{-1}·mg^{-1} dry weight for the maximally active dog heart (46)). Relative to isolated mitochondria, a staggering 100-fold increase of the extracellular p_{50} is measured in heavily stirred permeabilized fiber bundles prepared from rat heart and soleus muscle (39), in which case oxyconformance extends up to air saturation in terms of a monophasic hyperbolic oxygen dependence (Figure 6). Fatigue is accelerated in skeletal muscle fibers of the frog at an oxygen pressure of 4 kPa (30 mmHg (60)) which may fall into the region of initial diffusion limitation (Figure 6B). Similarly, oxygen conformation up to air saturation in superfused frog sartorius muscle is subject to diffusion limitation, although metabolic suppression mediated by signals triggered by a cellular oxygen sensor may always be difficult to exclude (5). Increased diffusion distances are in line with the distinct kinetic responses to external oxygen, when highly oxygen-independent fibroblasts and endothelial cells are compared with oxyconforming cardiomyocytes and fiber bundles (Figs. 4 and 6), spanning a 0.1·10^6-fold volume range (Table 1).

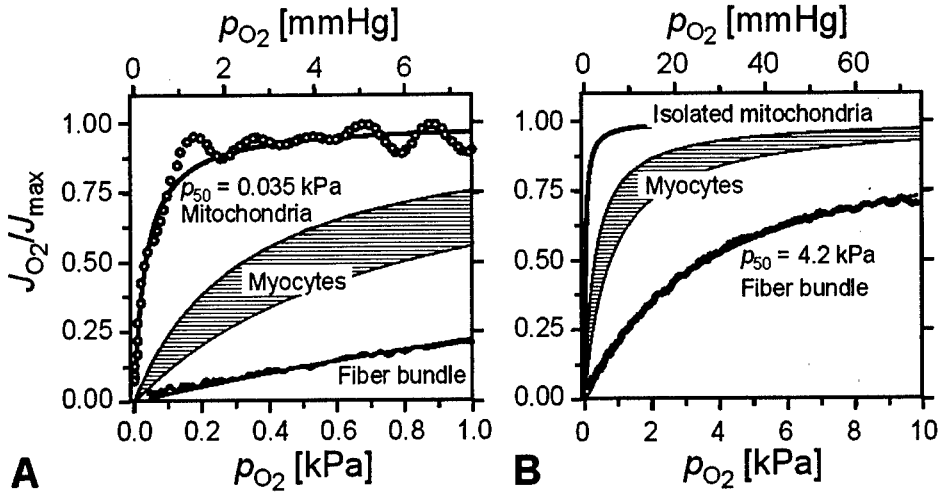

Figure 6. Hyperbolic relation of mitochondrial active respiration, JO_2, and oxygen pressure, pO_2, in isolated heart mitochondria at state 3 (stimulated by ADP; after Gnaiger *et al.* (25)); isolated cardiomyocytes (resting (36) and stimulated by uncoupling (51)) and permeabilized rat skeletal muscle fibers (*M. soleus*) at state 3 (stimulated by ADP; after Kuznetsov *et al.* (39)). **A** and **B** show different ranges of oxygen pressure.

Table 1. Schematic geometry of cell systems with increasing oxygen diffusion limitation and oxyconformance, expressed as Δp_{50}, the difference between extracellular and mitochondrial p_{50}.

Cell	Shape	Radius	Length	Volume	Δp_{50}	
		μm	mm	nL	kPa	mmHg
Endothelial cell	Spherical	6.8	0.014#	0.0013	0.01†	0.1
Cardiomyocyte	Cylindrical	10	0.1	0.03	0.3-0.8‡	2-6
Fiber bundle	Intertwined	150	2	140	1.4-6.8§	10-50

Diameter and radius of suspended human umbilical vein endothelial cells (26), calculated according to measured volume (33).

† From ref. (61).

‡ Radius and length from ref. (35); range of Δp_{50} from ref. (36, 51).

§ Radius and length from ref. (52); fiber bundels are intertwined in the stirred respirometer chamber; range of Δp_{50} is the mean ± SD from ref. (39).

HYPOXIA AND DOWNREGULATION OF ENERGY DEMAND

Matching of energy demand with energy supply is the prerequisite for homeostatic control of the cellular energy state. Respiration of adult cardiomyocytes becomes diffusion limited below pO_2 values of c. 2 kPa (Figure 7A). Hence induction of anoxic tolerance in cultured adult rat cardiac myocytes by conditioning at 1 % O_2 (1 kPa) (57) is possibly mediated by its effect on mitochondrial function. Hypoxia is partly compensated by increased glycolytic ATP production and accompanied by reversible downregulation of contractile activity (62, 63). An entirely different response is observed in chick embryonic cardiomyocytes (9), which have a 100-fold lower oxygen consumption per cell compared to adult rat cardiomyocytes, and present a much higher degree of oxyconformance which is unrelated to diffusion restriction (Figure 7B). Vertrebrate and particularly bird embryo hearts develop normally in a low-oxygen microenvironment and display low oxidative metabolism. Vascularization and myoglobin are absent in early developmental stages when cardiac function is less oxygen dependent and anoxic tolerance is relatively high (55). Experimental conditions well below air saturation (Figure 7B), therefore, mark the transition from hyperoxia to hypoxia. It remains to be defined, how low the pO_2 needs to be set in the incubation medium to provide a "normoxic" environment for embryonic cardiomyocytes.

The respiratory response of beating embryonic (9) and resting neonatal cardiac cells (11) is immediate, and thus independent of hypoxic conditioning (Figure 7B). It is tempting to interpret the onset of microxic regulation (21) of the neonatal cardiomyocytes (Figure 7B; arrow) as somewhat intermediate between the oxygen response pattern of embryonic and adult heart cells. The proton permeability in neonatal mitochondria is higher than in adult cardiac mitochondria (67). Importantly, at least part of the oxyconformance in neonatal cardiomyocytes is caused by suppression at low oxygen of the proton leak component of oxygen consumption not coupled to ATP synthesis (12). In addition to the inhibition of non-COX respiration (Figure 5), this contributes to an increased biochemical efficiency of ATP production per unit oxygen consumed at low oxygen levels, as supported by studies on isolated mitochondria (27).

Rather than generalizing pO_2-dependent downregulation of respiration and ATP utilization, the striking differences in various developmental stages of cardiac cells warrant explanation. Discussing these different physiological responses to oxygen pressure in cardiomyocytes from mammals and birds at different developmental stages under the umbrella of short-term "hibernation" (10-12, 57, 63) draws attention to the importance of homeostatic control of ATP demand in the face of changes in supply. The adaptive mechanisms of metabolic downregulation in hypometabolic states of hypoxia (31), however, are more clearly appreciated by relating physiological and biochemical control mechanisms to the diversity of oxygen regimes and metabolic challenges met by various types of mitochondria, cells, tissues and organisms.

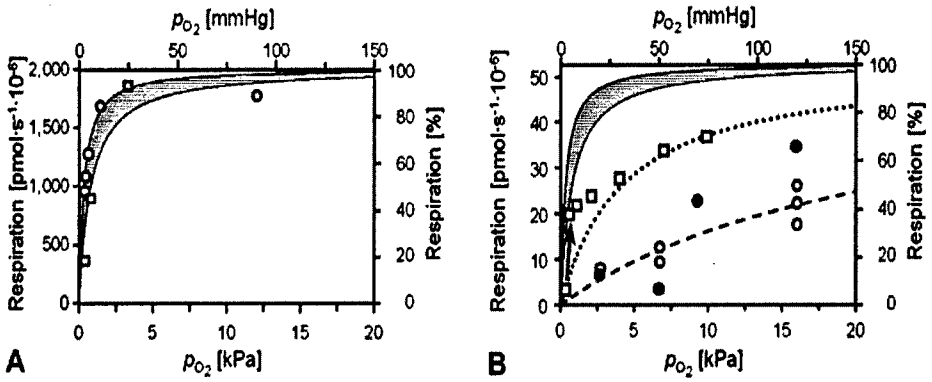

Figure 7. Oxygen dependence of respiration in adult (A) and neonatal or embryonic cardiomyocytes (B). **A.** Circles and squares: data from Stumpe and Schrader (62, 63); activated cardiomyocytes in an oxystat system (left *Y*-axis; based on 4 mg protein per 10^6 cells (57, 71)). The small degree of apparent oxyconformance is within the range of cellular diffusion limitation. **B.** Squares: resting neonatal rat cardiomyocytes; data from Casey and Arthur (11); strongly biphasic (arrow), in contrast with a hyperbolic oxygen dependence (dotted line; permeabilized myocardial fiber bundles with p_{50} of 4.2 kPa; right *Y*-axis). Circles: acute or prolonged (open) and sustained (closed) exposure to various oxygen levels in beating chick embryo cardiomyocytes; data from Budinger *et al.* (9) (dashed line: hyperbolic trend line for open circles with apparent p_{50}>20 kPa). Adult cardiomyocyte oxygen kinetics is shown as a common reference in both panels as the hatched area, lower boundary line: p_{50} of 0.32 kPa (2.4 mmHg (36), upper boundary line: 0.79 kPa (5.9 mmHg (51)) for resting and uncoupled cardiomyocytes (right *Y*-axes).

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Chapter 5

CURRENT PARADIGMS IN CELLULAR OXYGEN SENSING

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Abstract: Organisms, tissues and cells react to hypoxia by activating adaptive responses that tend to preserve systemic oxygen transport, cellular oxygen delivery, and the resistance of cells against the consequences of severe hypoxia. These responses are required for embryonic development and for survival through adulthood. Although much has been learned about the signaling pathways that are activated in hypoxic cells, the underlying mechanism of O_2 sensing is not established. Most of the putative models of O_2 sensing include the involvement of redox-dependent reactions and many implicate reactive oxygen species in the signaling process. The sources of these oxidant signals are thought to include members of the NAD(P)H oxidase system and/or mitochondria. This article reviews evidence for and against the involvement of these systems in the O_2 sensing pathway.

Key Words: reactive oxygen species; hypoxia, mitochondria, NAD(P)H oxidase

INTRODUCTION

Mammalian species rely on molecular oxygen to support mitochondrial oxidative phosphorylation, which is required for survival. When cellular oxygen tensions fall below a critical level, mitochondrial ATP production may decrease if the availability of O_2 at the terminal cytochrome oxidase limits the electron flux through the respiratory chain (81). When that situation occurs, organ system function cannot be sustained because cellular energy stores are limited. Consequently, the survival of the organism becomes threatened. To prevent the onset of that situation, multicellular organisms have developed a complex set of adaptive mechanisms that function to assure a continued supply of O_2 under a wide range of physiological and environmental conditions.

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Adaptive mechanisms that protect cellular oxygen delivery are evident at the organismal level, at the organ system level, and at the microvascular level. At the organismal level, peripheral chemoreceptors can detect a decrease in arterial O_2 tensions and trigger an increase in alveolar ventilation that can improve systemic oxygen delivery (54). Simultaneously, chemoreflex-mediated increases in sympathetic autonomic activity tend to limit blood flow and oxygen delivery to tissue regions with lesser oxygen needs, thereby preserving oxygen supply to tissue regions with greater metabolic demands. At the microvascular level, recruitment of perfused capillary density allows tissues to extract a greater amount of O_2 from a limited delivery, helping to match cellular oxygen delivery to cellular metabolic requirements (63).

If systemic hypoxemia persists, additional mechanisms are activated including erythropoiesis and angiogenesis. The former response is mediated primarily by an increase in the synthesis and release of erythropoietin from cells in the kidney and liver, and results in an increase in the oxygen carrying capacity of blood. The latter response is mediated by the release of vascular growth factors from parenchymal cells, and results in the growth of capillaries into hypoxic tissue regions. Both of these responses effectively increase the transport of oxygen from the lungs to the cells of the body. Collectively, these adaptive mechanisms reflect integrated organ system responses to systemic hypoxia and they require an increased metabolic activity among the effector cells despite the overall decrease in systemic oxygen availability (65).

Individual cells have also acquired the ability to activate a variety of adaptive mechanisms that allow them to protect themselves from the consequences of oxygen deprivation. In response to hypoxia, many cells increase the transcriptional activation of enzymes involved in glycolysis, membrane glucose transporters, vascular growth factors including vascular endothelial growth factor (VEGF), and other genes that confer protection against the consequences of cellular anoxia. The upregulation of these genes is achieved by the activation of a small number of transcription factors including Hypoxia Inducible Factor (HIF-1 or -2), Nuclear Factor kappa B (NF-kB), Activator Protein -1 (AP-1) or the tumor suppressor factor p53 (20, 55, 64). Through non-transcriptional mechanisms, some cells have the ability to suppress metabolic activity during hypoxia, thereby lessening the local depletion of O_2 in the tissue and possibly protecting the cell in the event that more severe hypoxia ensues.

Regardless of whether adaptive responses occur at the organismal level or at the molecular level, a primary requirement in each of these responses is the need to detect cellular hypoxia. Moreover, cells must be capable of detecting encroaching hypoxia well before it becomes a critical threat to survival, because most of these adaptive responses require some time to develop and many are intended to preserve or augment the supply of oxygen to the tissues. More importantly, an adaptive response that is not triggered until tissue anoxia has developed is of little use in preventing that condition in the first place. Although certain specialized cells such as the chemoreceptive type I cells of the carotid body are well known for their ability to sense oxygen, virtually every cell has the ability to appear to detect the onset of hypoxia and to trigger hypoxia-dependent responses. This property was first demonstrated by Ratcliffe and colleagues who transfected a plasmid consisting of the HIF-1-responsive promoter region of the erythropoietin gene (HRE) tied to a reporter gene (LacZ) into fibroblasts, a cell line not known to possess O_2 sensing properties (46).

They found increased expression of the reporter gene when the cells were made hypoxic, revealing that the cells were able to detect hypoxia and to activate transcription in response to a decrease in O_2 levels.

Since that time, great progress has been made in identifying the transcription factors activated in response to hypoxia, and in clarifying the signal transduction sequences by which they activate specific genes (31, 66). However, the identity of the underlying O_2 sensing mechanisms has remained, for the most part, a mystery. The aim of this review is to provide a critical examination of the various O_2 sensing mechanisms that are currently being advanced by different groups.

CURRENT MODELS OF OXYGEN SENSING

A central question in the field of oxygen sensing is whether a single oxygen transduction process exists, or whether different mechanisms are operative in cells of various tissues. Many investigative groups have focused on a particular oxygen-sensitive cell type such as the carotid body type I cells, vascular smooth muscle cells, pulmonary neuroepithelial bodies, or the hepatoma cell lines that express erythropoietin in response to hypoxia. A number of different models have emerged from these studies, and the following sections examine the major models that are under current investigation.

NAD(P)H Oxidase

The NAD(P)H oxidases are a family of multi-subunit complexes that oxidize NADPH or NADH and generate superoxide by transfer of an unpaired electron to O_2. The best known member of this family is the NADPH oxidase responsible for the respiratory burst in phagocytic cells (7). In phagocytic cells, NADPH oxidase assembly occurs at the plasma membrane and secretion of superoxide occurs into the extracellular space or into phagosomes of ingested pathogens. A similar system has been identified in non-phagocytic cells including endothelium, smooth muscle cells and fibroblasts (27). That system is comprised of membrane-associated gp91phox and p22phox subunits that make up the cytochrome b_{558} heterodimer, and the cytosolic p40phox, p47phox and p67phox proteins. Other regulatory subunits have also been described, including the small GTPase proteins rac-1, rac-2 or rap1A (27). The rac proteins appear to play a role in the activation of NADPH, although the specific pathways responsible for their activation are not fully understood. In neutrophils the activation of the complex includes the phosphorylation of p47phox protein kinase C, which causes translocation of the cytosolic subunits to the membrane (18), but the activation of the non-phagocytic enzyme is more complex (67). Other cell types also express components of the NADPH oxidase system. This oxidase has been demonstrated to function as a required component in the signal transduction cascade leading to hypertrophy in response to angiotensin II in vascular smooth muscle cells. Like the phagocytic form, the vascular smooth muscle NAD(P)H oxidase contains the p22phox and p47phox subunits, but the gp91phox is replaced by Nox-1 and Nox-4 subunits (39, 69). Rac appears to contribute to the activation of the oxidase in response to angiotensin II in smooth muscle cells (67). Like the phagocytic form of the enzyme, activation of the non-phagocytic enzyme complex is associated with its translocation to a membrane. However, this may involve

intracellular membranes rather than to the plasma membrane, resulting in the release of superoxide within the cell rather than to the extracellular space. It is also possible that the non-phagocytic enzyme localizes to the plasma membrane but releases superoxide to the cytosolic compartment.

The putative role of NAD(P)H oxidase in O_2 sensing is thought to relate to changes in the rate of reactive oxygen species (ROS) generation in response to changes in the cellular O_2 tension (37). Because the oxidase uses O_2 as a substrate, it has been proposed that decreases in cell PO_2 cause a progressive shift in the intracellular oxidation-reduction (redox) conditions to a more reduced (i.e., less oxidized) state as the activity of the oxidase declines. Evidence in support of a role for NAD(P)H oxidase in oxygen sensing includes the observation that subunits of the enzyme are expressed in cells known to participate in the oxygen sensing response (25). Moreover, some studies have reported finding decreases in the production of oxidants during hypoxia (4), while other studies have found that inhibitors of superoxide dismutase enhance the hypoxic response, presumably by decreasing H_2O_2 production (1).

A member of the NAD(P)H oxidase family described as an NADH oxido-reductase was reported by Mohazzab-H. *et al.* to play a role in the O_2 sensing responsible for hypoxia pulmonary vasoconstriction (HPV) in pulmonary arteries (49). Burke and Wolin found that H_2O_2, possibly released by that oxidase, caused relaxation of preconstricted pulmonary arteries along with activation of guanylyl cyclase (10). They suggested that vasodilation during normoxia was mediated by an H_2O_2-catalase complex, which was thought to activate guanylyl cyclase. According to this model, high levels of H_2O_2 generated during normoxia would tend to keep catalase in an oxidized state (termed Compound I), leading to greater activation of guanylyl cyclase. By contrast, during hypoxia the prevalence of Compound I would decrease as the production of H_2O_2 declined, leading to the loss of vasodilator influence and subsequent increase in contraction. A concern with this model is that intracellular antioxidants, or inhibitors of the oxidoreductase, should produce sustained pulmonary vasoconstriction because they would mimic the effects of low PO_2. However, this response has not been reported.

One cell system that appears to require NAD(P)H oxidase for the O_2 sensing response is in neuroepithelial bodies (NEB) in the airway mucosa of various species, including humans (83). These neuroendocrine cells contain components of the NAD(P)H oxidase system (82) are thought to function in some capacity as airway chemoreceptors, based on their morphological similarity to carotid body type II cells. In isolated neuroepithelial cells, hypoxia elicits degranulation (40), and patch-clamp studies have identified changes in membrane voltage-dependent potassium channels during hypoxia (50, 51). There is convincing evidence linking the NADPH oxidase system to the O_2-sensitive responses in NEB cell. Fu *et al.* (22) performed patch clamp experiments on NEB cells in fresh lung slices from wild-type and gp91phox knockout mice. During hypoxia (15-20 mmHg) they observed an inhibition of both Ca^{2+}-dependent and –independent K^+ currents in wild type but not the oxidase-deficient mice. Diphenylene iodonium (DPI), which inhibits flavoproteins including the oxidase, decreased the K^+ current in wild type but not the knockout cells. These results indicate a requirement for NADPH oxidase in the NEB response to hypoxia. However, they do not clearly establish whether that oxidase system functions as the O_2 sensor or whether it functions as a downstream amplifier of an upstream O_2 sensor.

While attractive in its simplicity, several observations raise concern about the involve-

ment of NAD(P)H oxidase as an O_2 sensor. First, the observation that subunits of the NAD(P)H oxidase system are expressed in O_2-sensitive cells does not necessarily mean that they participate in the oxygen sensing pathway. Second, some studies have reported finding an increase in ROS levels during hypoxia, rather than a decrease (45, 75). Furthermore, if decreases in ROS production trigger the functional response to hypoxia, then pharmacological inhibitors of NAD(P)H oxidase or cell-permeable scavengers of ROS should mimic the hypoxic response by attenuating the ROS signals. Yet pharmacological inhibitors such as diphenylene iodonium (DPI) that attenuate ROS production have been shown to block the response to hypoxia rather than to activate it during normoxia (38, 45, 71, 72, 75), while antioxidants block hypoxic responses rather than activating them (75, 76). Several studies have reported that ROS may augment contraction in pulmonary arteries (36, 58, 59) suggesting that an oxidant signal may underlie the hypoxic vasoconstriction response. Transgenic knock-out mice lacking the NAD(P)H gp91phox subunit exhibit a minimal phenotype and retain functional responses to hypoxia including hypoxic pulmonary vasoconstriction (5), indicating that the phagocytic form of the oxidase is not required for normal O_2 sensing. However, this observation does not rule out the possibility that other NAD(P)H oxidase family members might still be involved. Clearly, much controversy still exists regarding the potential role of this enzyme in the O_2 sensing response.

Mitochondria as Oxygen Sensing Organelles

Mitochondria have long been considered to be a potential site of oxygen sensing, based on the facts that they function as the primary site of O_2 consumption, and they are capable of binding oxygen with high affinity. Thus from a teleological standpoint, they represent an ideal site for O_2 sensing. On the other hand, several observations also speak against their involvement. First, the apparent Km of O_2 for cytochrome oxidase is less than 1 μM (11, 16, 60). While this allows mitochondria to sustain electron transport down to very low levels of oxygen tension, it would appear to prevent changes in mitochondrial redox until near-anoxic conditions were reached. If true, this would make mitochondria excellent sensors of anoxia but poor sensors of hypoxia. Second, a number of studies have failed to block responses to hypoxia using cyanide, an inhibitor of cytochrome oxidase (70, 76). Observations such as these have led some investigators to conclude that mitochondria are not required for the O_2 sensing mechanism (9).

However, more recent studies have reopened the question of mitochondrial involvement by proposing that the function of upstream electron transport complexes is required for O_2 sensing, whereas the distal electron carriers are less directly involved (12, 13). Two current paradigms involving mitochondrial involvement in O_2 sensing both involve the production of reactive oxygen species (ROS), which appear to act as signal transduction messengers.

Mitochondria have been known to generate reactive oxygen species for many years (8). Until recently, these were merely thought to represent toxic byproducts of the electron transport process. Superoxide is generated when molecular oxygen accepts a single unpaired electron. Such accidental transfer of electrons to O_2 can potentially occur at multiple sites in mitochondria (Figure 1). The electron transport chain consists of complexes that mediate the transfer of electrons along a pathway with increasing standard redox potentials. Molecular oxygen has a strongly positive standard potential relative to all of those carriers, so it is capable of snatching unpaired electrons from multiple sites along the chain. Most

likely sites of superoxide generation include iron-sulfur centers (Complexes I, II and III), flavin groups (I and II), and especially the ubisemiquinone site. That compound itself is a free radical component of the Q cycle, which couples Complexes I and II with Complex III. Most of the reactive species generated by mitochondria are degraded, beginning with the dismutation of superoxide radicals by manganese superoxide dismutase (Mn-SOD) in the mitochondrial matrix and by Cu,Zn-SOD in the cytosol and mitochondrial intermembrane space. Hydrogen peroxide generated by SOD is subsequently degraded by the glutathione peroxidase systems in the cytosol or mitochondria, or to a lesser extent by catalase. Efficient degradation of superoxide is required to prevent oxidative damage to the cell; this point is demonstrated by the lethal phenotype in transgenic mice with homozygous deletion of the gene encoding Mn-SOD (42, 43).

Mitochondrial inhibitors such as rotenone, DPI, myxothiazol, antimycin A and cyanide block electron transfer at distinct sites, and can attenuate or augment ROS generation by affecting the flux of electrons into the different sites that can generate superoxide. For example, rotenone blocks electron transfer from Complex I into Complex III, thereby attenuating superoxide generation at the latter but potentially increasing its production at the former. Antimycin A, by preventing the degradation of ubisemiquinone, tends to augment ROS generation by prolonging the lifetime of that free radical. By virtue of their orientation and location in the inner membrane, the superoxide produced at various complexes can be released from the matrix side of the inner membrane, or alternatively in can be released from the outer surface of the inner membrane into to the intermembrane space (30). If mitochondrial ROS need to reach the cytosol in order to participate in cell signaling, it is reasonable to speculate that ROS generated on the outer surface of the inner membrane are more likely to be the source of these signals.

Superoxide and hydrogen peroxide are potentially useful as signaling molecules because they are chemically reactive, but not excessively so. This property allows them to be generated at one locus, and to oxidize a target molecule that is nearby but not immediately adjacent to the source. By analogy, nitric oxide, which acts as a signaling molecule in both an autocrine and a paracrine manner, would not be especially useful if it were so reactive that it oxidized the first molecule it collided with. By contrast, hydroxyl radical is generally useless as a signaling molecule because its lifetime ($\sim 10^{-9}$ sec) is so short that it is unlikely to reach its intended destination unless the target happens to sit immediately adjacent to the site of generation.

The involvement of mitochondrial ROS as signaling agents in the O_2 sensing response can potentially explain why cyanide failed to abolish the response to hypoxia. If ROS are generated from Complex I, II or III, then inhibiting electron transport at more distal sites in the electron transport chain should not prevent the generation of ROS at the more proximal sites. Furthermore, inhibition with cyanide could augment ROS generation by causing those proximal sites to become more fully reduced.

In regard to the role of ROS in the oxygen sensing function of mitochondria, two related but opposing theories have emerged. One view is that ROS generation by mitochondria decreases during hypoxia due to the lessened abundance of O_2 as a substrate for superoxide formation. The opposing model states that ROS production paradoxically increases during hypoxia, and that the increase in oxidant stress triggers a signal transduction sequence that ultimately results in the increase in Ca^{2+} that triggers contraction. Both models implicate the proximal region of the electron transport chain, and both invoke the participation of

ROS. Interestingly, in many cases the experimental findings have been similar. However, the fundamental question of whether mitochondrial ROS production increases or decreases with hypoxia has not been definitively resolved. The following sections review the currently opposing arguments, in the context of the hypoxic pulmonary vasoconstriction (HPV) response. The ability to constrict in response to hypoxia can be demonstrated in the isolated lung, in rings of pulmonary artery, and in isolated pulmonary artery smooth muscle cells. The HPV response therefore represents a physiological downstream response that is useful for considering the mechanism of O_2 sensing.

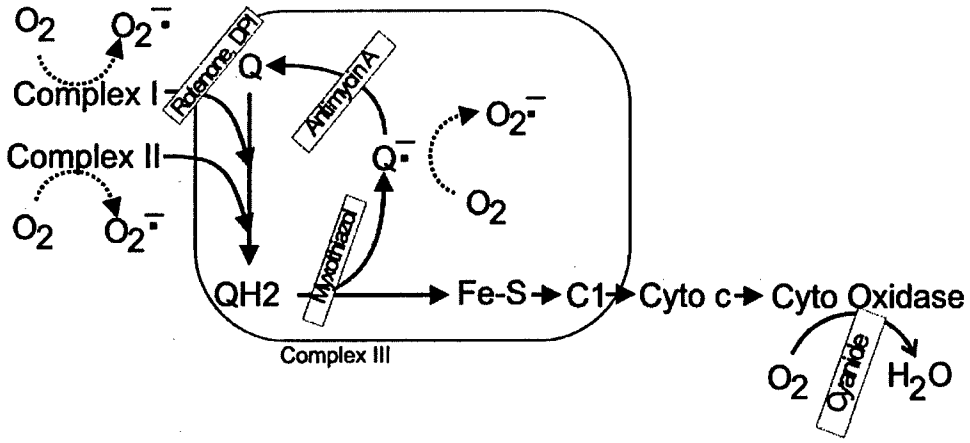

Figure 1. Schematic diagram of the mitochondrial electron transport chain. Sites of inhibition by electron transport inhibitors are shown in boxes. O_2^{-}: superoxide anion; Q^{-}: ubisemiquinone.

Does Hypoxia Decrease ROS Generation?

According to this theory, decreases in ROS generation during hypoxia produce a shift in cellular redox toward the reduced state, which in turn signals ion channels or other targets through the direct modulation of redox-sensitive thiol groups (56, 57). The rationale behind this model is based on the observation that lung mitochondrial ROS production increases in proportion to oxygen tension during hyperoxic ventilation (21). According to this theory, basal production of H_2O_2 during normoxia acts on redox-sensitive cysteine residues in voltage-dependent membrane potassium channels (3), causing the channels to remain open. This tends to hyperpolarize the plasma membrane of smooth muscle cells, preventing the entry of Ca^{2+} through voltage-dependent channels. During hypoxia, decreases in H_2O_2 production are hypothesized to result in the reduction of thiol groups on the channels, resulting in an inhibition of outward potassium current. This promotes membrane depolarization and subsequent opening of voltage-dependent calcium channels resulting in the activation of contraction. The source of the redox signals could be either NAD(P)H or mitochondria, either of which are may generate a basal oxidant stress during normoxia (80), which opens the K^+ channel by acting at regulatory thiol groups and thereby keeps vascular tone low. In

support of this theory, Vega-Saenz de Miera and Rudy (74) reported that 0.5-1.8 mM H_2O_2 administration to recombinant voltage-gated K^+ channels blunted the fast inactivation in response to a depolarizing stimulus. Conversely, oxidizing agents such as t-butyl hydroperoxide and diamide increased potassium currents (79).

This model has been studied using inhibitors of the electron transport chain. Proximal inhibitors such as rotenone and antimycin A were reported to decrease chemiluminescence (ROS) and to increase pulmonary artery (PA) pressure in response to hypoxia (3, 4) and to abolish subsequent responses to hypoxia. Interestingly, cyanide, an inhibitor of the distal end of the electron transport system, increased ROS production and PA pressure but did not abolish subsequent hypoxic responses.

In the pulmonary circulation hypoxia causes constriction, whereas in the systemic circulation hypoxia produces a local vasodilation. Michaelakalis *et al.* explain these differences by proposing that mitochondria in systemic and pulmonary vessels behave differently. Specifically, they reported that hypoxia increased ROS production in renal artery but decreased it in pulmonary artery (48). The Complex I inhibitor rotenone decreased ROS production in the lung vessels, whereas it increased ROS production in renal arteries. This interesting observation raises the mechanistic question of how mitochondria in different tissues would behave so differently in terms of ROS response to hypoxia.

An aspect of this model that is controversial relates to the notion that mitochondria generate a basal oxidative signal under normoxic conditions. In fact, cytosolic redox conditions in cells normally exist in a highly reduced environment (35, 53). Although oxidized (i.e., disulfide) bonds frequently serve as important components to extracellular proteins (23), the intracellular correlates of these proteins exist in the reduced (-SH) state. Consequently, a number of redox-regulated systems are activated in response to an oxidizing stimulus and suppressed by reductive conditions (6). Lucigenin, a chemiluminescence compound used to document decreases in ROS during hypoxia (48) are preferentially sensitive to extracellular oxidants and may not be sensitive to intracellular ROS signals. Other signaling pathways activated in response to membrane receptor-ligand interactions, such as the angiotensin II signaling pathway, utilize increases in ROS production to trigger responses (26, 27, 67). The levels of oxidants produced in response in these pathways are small, and are likely to be limited to specific subcellular compartments. Future studies are therefore required to establish more clearly the sources of ROS and their response to hypoxia.

Does Hypoxia Increase Mitochondrial ROS Production?

Recent studies support an alternative model of oxygen sensing involving increases in mitochondrial ROS production during hypoxia (78). In the pulmonary circulation, Waypa *et al.* found that electron transport inhibitors rotenone, DPI and myxothiazol, which block electron transport into Complex III, each selectively blocked the response to hypoxia without inhibiting the vasoconstriction in response to U46619, a thromboxane A2 analog (75). In contrast to the results of Archer *et al.* (3), these proximal mitochondrial inhibitors produced minimal changes in PA pressure during normoxia. The HPV response was also selectively inhibited by the antioxidant compounds pyrrolidine dithiocarbamate, a thiol reductant, and ebselen, a glutathione reductase mimetic drug. By contrast, mitochondrial inhibitors that block electron transport at more distal locations (antimycin A or cyanide) failed to inhibit the hypoxic response and actually increased in PA pressure during nor-

moxia, which is consistent with their known ability to increase mitochondrial ROS production (73). Hypoxia caused an increase in the oxidation of 2',7'-dichlorofluorescin diacetate (DCFH) in cultured PA myocytes, and this response was attenuated with myxothiazol. These findings suggested that hypoxia augments ROS production in mitochondria, and that this response is required for the increases in Ca^{2+} that mediate contraction. Parallel studies in cultured PA smooth muscle cells provided results consistent with those shown in the whole lung. In both experimental systems, exogenous H_2O_2 caused contraction, rather than relaxation, during normoxia. Using isolated intrapulmonary arteries, Leach *et al.* also found that inhibition of Complex I and III abolished HPV and calcium activation without causing normoxic vasoconstriction (41), consistent with a requirement for electron transport and increased ROS in the HPV response. In another study, Waypa *et al.* found that the proximal region of the electron transport chain was required for the increases in cytoplasmic Ca^{2+} during hypoxia, and that overexpression of catalase in PA smooth muscle cells attenuated the Ca^{2+} response to hypoxia or H_2O_2 without altering the response to angiotensin II. An interesting speculation is that H_2O_2 released from mitochondria may trigger Ca^{2+} release through activation of ryanodine receptors via oxidation of cysteine thiols on the channel (52, 68). If so, then activation of Kv channels in HPV might represent a later amplification step rather than an initiating event. In either case, to date, three separate groups have reported finding evidence of increased ROS production in PA myocytes during hypoxia (24, 28, 29, 45, 77).

Recently, a fourth group has found evidence of increased ROS production in PA myocytes during hypoxia. In an elegant study (44), Liu *et al.* used DCFH, lucigenin-enhanced chemiluminescence, and electron paramagnetic resonance (EPR) spectroscopy to detect ROS production during hypoxia. In small pulmonary arteries subjected to moderate hypoxia, they found decreased diameter (i.e., constriction), increases in DCF fluorescence, a trend toward increased lucigenin chemiluminescence, and EPR spin trap evidence of hydroxyl and/or alkyl radical production, which was attenuated or abrogated by SOD + catalase (CAT). The SOD + CAT also blocked the increase in DCF fluorescence during hypoxia. They concluded that HPV requires an increase in ROS production within smooth muscle cells of the pulmonary artery.

An increase in mitochondrial ROS production has also been observed in liver cells (12), cardiac myocytes (17), endothelial cells (2), tumor cell lines (13, 14), and other cell types (13). In addition to mediating the HPV response, there is evidence to suggest that the increase in ROS during hypoxia is required for the stabilization of HIF-1 (12, 13), NF-kB (14), and p53 (15) transcription factors. The diversity of these responses leads to the speculation that mitochondria may play a broader role in mediating cellular responses to hypoxia. An attractive possibility is that mitochondria may function as a "unifying mechanism" of oxygen sensing.

Against this theory is the recent evidence identifying a role for prolyl hydroxylase in the HIF-1a stabilization pathway. HIF-1 is a heterodimeric transcription factor whose alpha and beta subunits are constitutively expressed (64). Activation of HIF during hypoxia is regulated primarily at the posttranscriptional level. During normoxia, the alpha subunit is rapidly degraded by the ubiquitin-proteasomal system (31), whereas the beta subunit remains stably expressed at the protein level. During hypoxia, the degradation pathway is inhibited allowing the alpha subunit to accumulate, to heterodimerize with the beta subunit, and to activate transcription. The signal for degradation of the alpha subunit during nor-

moxia begins with its hydroxylation at a highly conserved proline residue, by a prolyl hydroxylase (32-34). The hydroxylation facilitates the interaction of the protein with pVHL, the E3 ubiquitin ligase responsible for polyubiquitin tagging (47). Therefore, a key initiating step in the hypoxic stabilization of the protein involves inhibiting the activity of prolyl hydroxylase. Interestingly, that enzyme is a dioxygenase that requires O_2 as a substrate for the hydroxylation step (19). This has led to the speculation that prolyl hydroxylase itself is the O_2 sensor responsible for HIF-1 activation (84), as its activity might become limited at low O_2 tensions. While this possibility cannot be ruled out at present, the experiments evaluating the activity of the hydroxylase under hypoxia were actually carried out under near-anoxic conditions (34). Since the enzyme cannot function under anoxic conditions, this is not an adequate test of its ability to regulate hydroxylation within the physiological range of O_2 tensions. Interestingly, ROS production must also halt during anoxia because O_2 is no longer available to generate superoxide. An adaptive response such as HIF-1 activation needs to be sustained even if the tissue approaches anoxia. Hence, it is interesting to speculate that ROS are required to initiate the HIF-1 response during hypoxia, and that the response is sustained in the absence of ROS during anoxia by the inability of prolyl hydroxylase to continue to function (61, 62).

SUMMARY

Oxygen sensing is a fundamental response that is required for embryonic development, for normal tissue function, for adaptation to environmental hypoxia, and for pathological processes including tumor growth. Despite its importance, the underlying mechanism by which cells detect a fall in O_2 tension and activate protective mechanisms has not been identified. Current theories regarding O_2 sensing mechanisms include NAD(P)H oxidases that increase or decrease ROS production in response to changes in PO_2, mitochondria that increase or decrease ROS production in an O_2-dependent manner, prolyl hydroxylase, heme proteins, and other systems. It is tempting to speculate that a single unifying mechanism of oxygen sensing might exist within cells, although the identity of that sensor is not yet resolved.

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Chapter 6

WHY IS ERYTHROPOIETIN MADE IN THE KIDNEY?

The kidney functions as a 'critmeter' to regulate the hematocrit

Sandra Donnelly

Abstract: The normal hematocrit is not a random number, but one that maximizes oxygen delivery. While the feedback loop wherein tissue oxygen pressure determines the production of erythropoietin, which further drives the production of red blood cells in the bone marrow, explains how the hematocrit is *generated*, it does not speak to how the hematocrit is *regulated*. The regulation of the hematocrit requires the coordination of the plasma volume and the red cell mass. By controlling red cell mass via erythropoietin and plasma volume through excretion of salt and water, the kidney is able to generate the hematocrit. It is hypothesized that the kidney functions as a *critmeter* by sensing the relative volumes of each component of the blood through the common signal of tissue oxygen tension. The kidney's unique ability to sense ECF volume through tissue oxygen signal allows it to coordinate these two volumes to produce the normal hematocrit. Hence, it may be the kidneys ability to report a measure of ECF volume as a tissue oxygen signal and thus to regulate the hematocrit that establishes it as the logical site of erythropoietin production. The *critmeter* is proposed to be a functional unit located at the tip of the cortical labyrinth at the juxta-medullary region of the kidney where erythropoietin is made physiologically. Renal vasculature and nephron segment heterogeneity in sodium reabsorption likely provides the anatomical construct to generate the marginal tissue oxygen pressure required to trigger the production of erythropoietin. The balance of oxygen consumption for sodium reabsorption and oxygen delivery is reflected by the tissue oxygen pressure. This balance hence determines RBC mass adjusted to plasma volume. Factors that affect blood supply and sodium reabsorption in a discordant manner may modulate the *critmeter*, e.g. angiotensin II. The objective of this work is to describe the hypothesis of the kidney's function as a *critmeter*, including the anatomical and physiological components, and the role of the renin-angiotensin system in modulating erythropoietin. Clinical examples of the dysregulation of the critmeter may be found in the anemia of renal failure and in sports anemia.

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Key Words: fractional sodium reabsorption, oxygen consumption, renin-angiotensin system, angiotensin II, chronic renal failure, sports anemia

INTRODUCTION

Erythropoietin is distinct amongst the hematopoietic hormones in that it is made remote from the bone marrow. The site of production is the adult kidney which begs the question, 'why the kidney?' The kidney regulates extra-cellular fluid (ECF) volume and plasma volume by regulating salt and water excretion. Further, the kidney regulates red cell mass by the production of erythropoietin. It may be the need to coordinate these two components of blood that establishes the kidney as the logical site of erythropoietin production.

The normal hematocrit is not a random number, but one that maximizes oxygen delivery (27). While the widespread understanding of the feedback loop wherein tissue oxygen pressure determines the production of erythropoietin which further drives the production of red blood cells in the bone marrow explains how the hematocrit is *generated,* it does not speak to how the hematocrit is *regulated.* The regulation of the hematocrit requires the coordination of the plasma volume and the red cell mass. It may be the kidney's unique ability to translate a measure of plasma volume into a tissue oxygen signal that operationally enables the kidney to regulate hematocrit. It may be that the kidney senses and adjusts the hematocrit and thus sub-serves the function of a *critmeter.*

The objective of this work is to describe the hypothesis that the kidney functions as a *critmeter.* The description includes the anatomical and physiological constructs and the role of the renin-angiotensin system in modulating erythropoietin production. Clinical examples of the resetting or dysregulation of the *critmeter* may be found in the anemia of renal failure and in sports anemia.

THE RENAL PRODUCTION OF ERYTHROPOIETIN AND THE KIDNEYS' FUNCTION AS A *CRITMETER*

The peri-tubular fibroblasts (4,49) of the renal cortex produce erythropoietin in response to tissue hypoxia (21,40,43,54,63). The number of interstitial fibroblasts staining positive for erythropoietin mRNA directly determines the rate of erythropoietin production (21) and its serum levels (40,66). Oxygen supply vs. demand regulates erythropoietin production in a feedback loop where tissue oxygen pressure is of central importance (Figure 1) (7,24,56). A fall in tissue oxygen pressure increases erythropoietin production. At the bone marrow, erythropoietin acts on both the burst forming units (BFU-E) and the colony forming units (CFU-E) for terminal differentiation. With the addition of new red cells, the red cell mass is increased which augments oxygen delivery to the tissues, thereby restoring normal tissue oxygen tension. This translates into a mathematical relationship between serum erythropoietin and hematocrit that is inverse logarithmic (10).

As in any tissue, renal tissue oxygen pressure is the net result of the rate of utilization of oxygen and the rate of oxygen delivery. Distinct from most other tissues where metabolic need determines blood flow, in the kidney, blood flow determines both sides of the equation

of oxygen supply and oxygen demand (Figure 1). Renal blood flow determines oxygen delivery, but as well determines the glomerular filtration rate (GFR). As it is the reabsorption of sodium that consumes ATP (13) and since 99% of filtered sodium is reabsorbed, the oxygen utilization is determined by the renal blood flow. Hence, the kidney is unique in that it is able to translate a measure of plasma volume into the metabolic signal of oxygen pressure. The tissue partial pressure of oxygen is likely the common parameter that coordinates the production of RBC by erythropoietin to match the plasma volume. Although it has been suggested that the location of an oxygen sensor in the kidneys controlling erythropoietin production "is most fortuitous" (25), a common location in the kidney is likely essential to coordinate the plasma volume and red cell mass.

On the other hand, it may seem counter-intuitive that the kidney should contain the oxygen sensor. The oxygen sensor should logically be located at a site that is sensitive to small changes in oxygen pressure. In spite of comprising 1% of body weight, the kidneys receive over 20% of the cardiac output (20). Renal oxygen consumption is only 8-10% of the delivered oxygen (31), suggesting that the renal oxygen supply is far in excess of need. Hence, the kidney would not appear to be a sensitive location for detecting hypoxemia. Notwithstanding the generous whole organ blood supply of the kidney, however, the heterogeneity of both the vascular anatomy and the function of nephron segments likely establish a marginal oxygen pressure at the tips of the cortical labyrinth at the cortico-medullary junction (Figure 2A). Firstly, there are relatively few arterial vessels found in the outer medulla and in the medullary rays (6). Secondly, somewhat like the countercurrent processes of the renal medulla, oxygen is shunted from the pre-glomerular arteries to the veins, resulting in cortical tissue oxygen pressures being less than venous oxygen partial pressure (62). Thirdly, nephron segment heterogeneity creates differences in metabolic demands of the nephron segments (31). For example, the metabolic cost of trans-epithelial sodium reabsorption varies along the length of the nephron. In the proximal tubule, one ATP is consumed per sodium reabsorbed while in the thick ascending limb, three ATP are used per sodium reabsorbed (31). In absolute terms, the amount of sodium reabsorption is at least three-fold greater in the proximal tubule where ATP production is obligatorily aerobic. Under physiological conditions, erythropoietin mRNA is found precisely in this juxta-medullary area of the kidney (21,43,44). Hence, the balance of oxygen supply and demand in this restricted area may be distinctly different than that of the whole organ, permitting the sensing of small changes in tissue oxygen pressure despite the apparent generous whole organ blood supply. With progressive degrees of anemia of non-renal origin or when other determinants of the delivery of oxygen become limiting, the partial pressure of oxygen decreases progressively and erythropoietin mRNA is found in evermore superficial areas of the renal cortex (Figure 2B).

It is hypothesized that the *critmeter* is a functional unit established by renal interstitial cells lying precisely between the tubular cells that consume oxygen and the capillaries that deliver oxygen (Figure 3). It would be found at the tip of the cortical labyrinth at the cortico-medullary junction in the kidney where erythropoietin is made physiologically. At this site, appropriate anatomical and physiological features may act in concert to establish this discreet area where oxygen supply approximates oxygen demand to generate the critical tissue oxygen pressure that triggers the production of erythropoietin under physiological conditions. As the oxygen consumption by the tubular cells is an indirect measure of plasma volume or filtered sodium, the interstitial cell is aptly sited to sense both plasma

volume as well as red cell mass components of the blood. This would enable the kidney to regulate the hematocrit and, thus to serve the function of a *critmeter*.

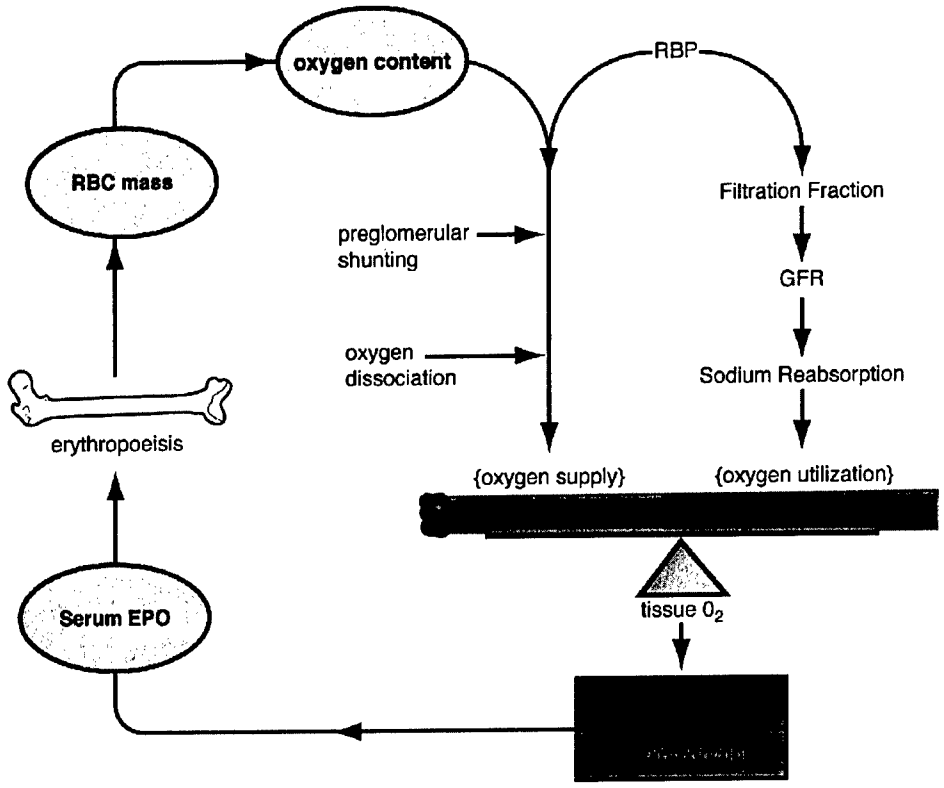

Figure 1. Production of erythropoietin. The rate of red cell production is adjusted in a feedback manner to the oxygen demand of peripheral tissues. Erythropoietin production is regulated by this feedback mechanism in which renal tissue oxygen pressure is of central importance. The supply of oxygen to the renal tissue is determined by the renal blood flow, the arterial oxygen content (which maybe be decreased at the renal tissue level compared to the renal artery by pre-glomerular shunting of oxygen) and the oxygen dissociation (which maybe be augmented at the renal tissue level by pre-glomerular shunting of CO_2 thus increasing the P50 of the hemoglobin saturation curve). The primary determinant of renal oxygen consumption is sodium reabsorption, which is largely determined by the GFR. In contrast to most tissues, RBF affects both the supply and the utilization of oxygen. The kidney is uniquely able to translate a measure of plasma volume into a metabolic signal of oxygen pressure and thus to coordinate the production of RBC by erythropoietin to match the plasma volume. In so doing, it is proposed that the kidney functions as a critmeter. (Used with permission from Elsevier: Reprinted from American Journal Of Kidney Disease, V38(2), Donnelly S, "Why is Erythropoietin Made in the Kidney? The Kidney Functions as a Critmeter", 415-425, 2001.

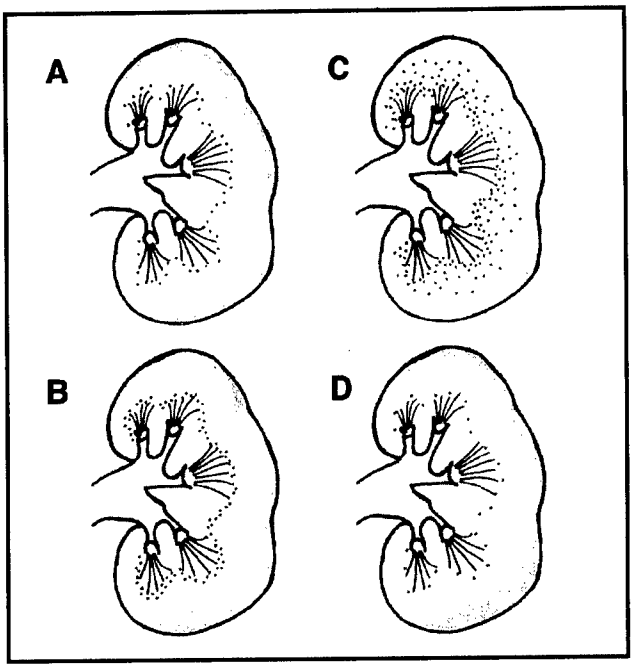

Figure 2. The critmeter and clinical examples of resetting and/or dysfunction of the critmeter. The erythropoietin mRNA is indicated by the dots and the partial pressure of oxygen is depicted in relative terms by the shades of gray (darker for higher partial pressures of oxygen). Figure 2A Under physiological conditions, the production of erythropoietin is confined to a small area at the tips of the juxta-medullary region of the cortical labyrinth. Figure 2B Non-renal anemia. With progressive degrees of anemia of non-renal origin or when other determinants of the delivery of oxygen become limiting, the partial pressure of oxygen decreases progressively and erythropoietin mRNA is found in evermore superficial areas of the renal cortex. Figure 2C Physiological regulation by the renin-angiotensin system. Due to the discordant effects of Ang II on the oxygen delivery and consumption, the partial pressure of oxygen may decrease at the critmeter with increased activity of the renin-angiotensin system and hence erythropoietin production. Figure 2D Renal anemia. As fractional sodium reabsorption decreases, renal tissue oxygen pressure increases to levels that exceed that needed to trigger the erythropoietin gene. (Used with permission from Elsevier: Reprinted from American Journal Of Kidney Disease, V38(2), Donnelly S, "Why is Erythropoietin Made in the Kidney? The Kidney Functions as a Critmeter", 415-425, 2001.

PHYSIOLOGY OF THE *'CRITMETER'* AND THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN MODULATING ERYTHROPOIETIN PRODUCTION

Renal tissue oxygen pressure most likely acts as the common signal integrating the relative amounts of the plasma volume and the RBC mass (Figure 3). Second messengers that translate the partial pressure signal may be a heme protein that has been demonstrated to be an oxygen sensor in the erythropoietin-producing hepatoma 3B cells in tissue culture

studies (29). Reactive oxygen species may also participate as a second method of hypoxia signal transduction (16). Other response elements to intracellular oxygen tension include hypoxia-inducible factor-1 (73) that interacts with the promoter of the erythropoietin gene in response to hypoxia to enhance transcription. As tissue culture of the renal erythropoietin-producing cell has defied efforts, the exact role of each of these factors in *renal* oxygen sensing awaits further study. However, tissue oxygen pressure remains central as the initial signal in our current understanding of the regulation of the production of erythropoietin in the kidney.

The balance between renal oxygen supply and demand ultimately determines the renal tissue oxygen pressure. Blood flow, hemoglobin, partial pressure of oxygen in the blood and the hemoglobin oxygen affinity curve influence oxygen supply. Notably, the partial pressures of oxygen and CO_2 delivered to the renal tissue may be lower or higher than systemic values respectively due to pre-glomerular shunting in the renal microvasculature (62). This augmentation of renal tissue pCO_2 may further affect the oxygen delivery indirectly by rightward shifting the hemoglobin-oxygen saturation curve. Eighty per cent of the kidney's oxygen consumption occurs in a direct relationship with sodium reabsorption (56). Sodium reabsorption by the proximal convoluted tubule modulates the production of erythropoietin (41) and inhibition of sodium reabsorption in the proximal tubule decreases erythropoietin production (22). Changes in plasma volume may also affect erythropoietin production (23). Factors that change the balance of oxygen supply and demand may modulate the tissue oxygen pressure and stimulate the production of erythropoietin in the restricted area of the cortex of the kidney.

The role of angiotensin II (Ang II) in modulating erythropoietin production can be considered as it is a hormone that produces an increase in sodium reabsorption (i.e. oxygen consumption) without increasing and indeed possibly decreasing renal blood flow (i.e. oxygen delivery). The renin-angiotensin system is modulated by a number of factors that ultimately control ECF (53,64). Renin affects renal sodium handling via Ang II, both indirectly by stimulating the production of aldosterone (a minor effect) and directly by effects on the kidney (37). Ang II constricts the efferent arteriole at the glomerulus, resulting in an increase in the filtration fraction. At the proximal tubule, Ang II increases transepithelial sodium reabsorption by stimulating the Na^+/H^+ exchanger. In addition, Ang II causes vasoconstriction of the vasa recta, thereby diminishing blood flow to the medulla. Thus, Ang II has discordant effects on oxygen supply (through effects on blood flow) and oxygen requirements (through effects on sodium reabsorption) and may thus influence the prevailing tissue oxygen pressure at the *critmeter* (Figure 2C).

Several experimental and clinical observations support a relationship between the renin-angiotensin system and erythropoietin production. Detailed studies of kidney function and sodium reabsorption assessed with standard clearance methods at baseline and during an infusion of Ang II were undertaken in healthy subjects. Serum erythropoietin levels increased by 24% at 24 hours (45). This effect of Ang II was completely abrogated when the subjects were pre-medicated with the Ang II receptor blocker losartan. In studies of shorter duration, changes in serum erythropoietin level correlated significantly with the change in filtration fraction in healthy subjects given losartan (18). Plasma renin activity is significantly higher in hemodialysis patients who do not require exogenous erythropoietin to maintain a hematocrit of approximately 30% compared to similar patients who do require recombinant human erythropoietin (rHuEpo) (67). Further, a doubling of

plasma renin activity induced by ultra-filtration is accompanied by a 69% rise in serum erythropoietin over 4 hours and this rise in erythropoietin is completely abolished by the use of angiotensin converting enzyme (ACE) inhibitors (67). Type 1 diabetic patients with hyporeninemic hypoaldosteronism and mild, if any, renal insufficiency have anemia due to erythropoietin deficiency (17). There is a direct correlation between plasma renin activity and erythropoietin in patients with normal renal excretory function who have glomerulonephritis or pyelonephritis (52). These data suggest an interaction of the two renal peptide hormones, erythropoietin and renin, in regulating both the absolute and the relative amounts of red cell mass and plasma volume, respectively.

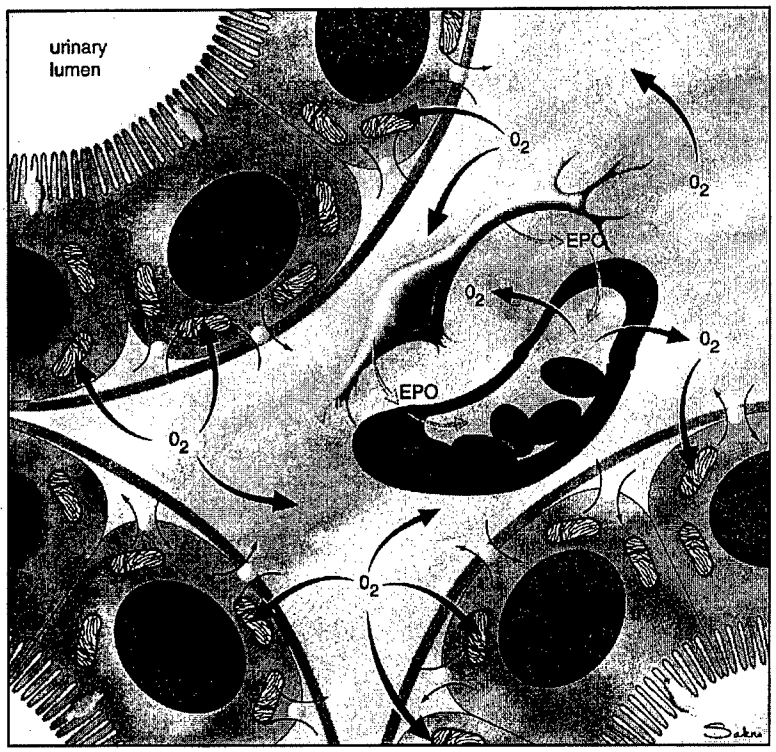

Figure 3. The critmeter and erythropoietin production. The erythropoietin producing cell in the renal interstitium likely senses the partial pressure of oxygen. At this site, the delivery of oxygen is determined by flow, hematocrit, blood pO_2 and the P50 of the hemoglobin. Notably, the partial pressure of oxygen of the blood delivered to the tissue may be less than mixed venous values due to pre-glomerular shunting. As well, the pre-glomerular shunting of CO_2 may increase the partial pressure and hence the P50 of the hemoglobin to facilitate oxygen delivery. The consumption of oxygen is determined by the trans-epithelial reabsorption of sodium which is directly related to the GFR and the filtered load of sodium and hence the ECF volume. Notwithstanding the importance of tissue oxygen pressure, the precise regulators of the erythropoietin-producing cell of the kidney remain undefined. (Used with permission from Elsevier: Reprinted from American Journal Of Kidney Disease, V38(2), Donnelly S, "Why is Erythropoietin Made in the Kidney? The Kidney Functions as a Critmeter", 415-425, 2001.

Clinical observations with the use of ACE inhibitors offer further support for the existence of a relationship. Patients treated with ACE inhibitors for hypertension experience an 8-10% fall in hemoglobin (30), despite normal renal excretory function. Diabetic patients taking ACE inhibitors have lower hemoglobin levels compared to diabetic controls (17). In patients with chronic renal failure, treatment with enalapril is associated with worsening anemia and decreased plasma erythropoietin (39). ACE inhibitors may cause anemia in hemodialysis patients who are not receiving rHuEpo (34,35) or attenuate the correction of anemia by rHuEpo (3,33,48). However, some conflicting data has been reported (1,14,15,69), the differences may lie in the relative doses of the ACE inhibitor and rHuEpo used or in the residual renal function of the patients. Finally, enalapril may cause anemia in renal transplant recipients treated for hypertension (38,68) or withdrawal of ACE inhibitors has been associated with polycythemia and thrombosis in a renal transplant patient (46).

From a teleological perspective, incorporating the production of erythropoietin in the feedback pathways that control and regulate blood volume provides a more comprehensive physiological loop. Circulating blood volume is regulated primarily by the neuro-hormonal signals generated in the brain stem in response to the afferent signals (Figure 4). Notably, the afferent signals derive from the volume receptors in the vena cava and the right atrium, as well as the pressure sensors in the aortic arch and the carotid sinus. Efferent signals in this loop affect primarily sodium balance through the sympathetic, renin-angiotensin and vasopressin systems and hence speak primarily to the plasma component of the blood. Incorporating the effect of the renin-angiotensin system on erythropoietin production completes the loop, such that the efferent signals speak to both of the components of blood volume represented in the afferent signals (Figure 4).

"WHY IS ERYTHROPOIETIN NOT MADE IN THE FAILING KIDNEY?"

The Anemia of Chronic Renal Failure

Although the anemia of renal failure results from a number of factors such as shortened red blood cell (RBC) survival, decreased marrow activity due to retained inhibitors in the uremic milieu and blood loss resulting from the qualitative platelet defect, an inappropriately low level of erythropoietin is central in its pathogenesis (26,50).

Anemia becomes a consistent feature of renal failure as the creatinine clearance falls below 40 mls/min/1.73m2 (12,36,55), but the etiology of the renal failure influences the degree of anemia. Erythropoietin deficiency has long been attributed to the decreased capacity of the kidney to make erythropoietin as renal failure progresses (26), but the reason the failing kidney makes inadequate erythropoietin remains poorly understood. Clearly a "structural" reason is responsible if interstitial fibrosis destroys the erythropoietin producing fibroblast-like cell of the renal interstitium or interferes with the transmission of signals for erythropoietin production. However, a decreased rate of production of erythropoietin as a *functional* consequence of the decline of the GFR has recently been considered (11).

Several lines of evidence support the notion that the failing kidney maintains the ability to make erythropoietin. In patients with chronic renal failure, serum levels of erythropoietin are in the normal range, albeit, inappropriately low for the prevailing hemoglobin (42) and the anemia worsens after bilateral nephrectomy (65). Furthermore, in patients with chronic renal failure, an acute hypoxic or hemorrhagic stress is associated with an increase in serum erythropoietin (12). Hemodialysis patients had an increase in erythropoietin when dialysis was carried out at 3450m above sea level compared to 420m at the base of the mountain (8). Finally, the erythropoietin causing polycythemia in kidney transplant recipients derives in large part from the native kidneys (2,47). This suggests that erythropoietin deficiency in some forms of renal disease is a functional aberration of the failing kidney rather than an absolute loss of erythropoietin producing cells.

The mechanism leading to the loss of erythropoietin production in progressive renal disease can be considered in the context of the *critmeter*. In chronic renal failure, decreased fractional sodium reabsorption (61) with the attendant diminished oxygen consumption increases renal tissue oxygen pressure (9). The observed to expected erythropoietin levels vary directly with fractional sodium reabsorption in type 1 diabetic subjects with mild renal insufficiency (17) and are strongly correlated with the fractional sodium excretion in chronic renal failure patients (11). In chronic renal failure, as the oxygen demand at the site of the *critmeter* diminishes, the oxygen supply may become relatively abundant and the tissue oxygen pressure may be elevated beyond that required to trigger the production of erythropoietin (Figure 2D). This imbalance could generate a *functional* deficiency of erythropoietin associated with chronic renal failure.

In summary, the deficiency of erythropoietin in some forms of chronic renal failure may be functional because failing kidneys can be prompted to make erythropoietin given the appropriate physiological stimuli. Further, in chronic renal failure, erythropoietin production declines in parallel with the fall in fractional sodium reabsorption. Hence, the critical oxygen balance at the *critmeter* may be dissipated as the fractional sodium reabsorption declines in chronic renal failure thus resulting in erythropoietin deficiency.

WHY IS ERYTHROPOIETIN NOT MADE IN A NORMAL KIDNEY?

The Case of Sports Anemia

The phrase "sports anemia" was coined in 1970 by Yoshimura's review of anemia in the setting of exercise. Less than normal hematocrits are seen in up to 6% of trained athletes (5) and further falls in hemoglobin may occur with increased intensity of training (19). Several factors may contribute to the lower hemoglobin seen in athletes. Proposed mechanisms include plasma volume expansion, intravascular hemolysis, iron deficiency and starvation (5). Volume expansion has been clearly documented to occur as individuals start training and may be as great as 38% in males and 18% in females suggesting that the fall in hemoglobin seen in these athletes represents a "pseudoanemia" (71,72). Indeed, red cell mass increased by 35% in the male athletes, suggesting the presence of a lower hematocrit instead of a true anemia. Erythropoietin levels in sports anemia are lower than would be predicted in response to anemia in healthy normal subjects (57,70).

The changes in extracellular fluid volume and red cell mass as a physiological adaptation to exercise are complex and dependent on the intensity and duration of exercise. Plasma volume expansion is associated with increase sodium reabsorption, not only during the exercise period, but increase renal sodium avidity is demonstratable 24 hours after the exercise (51). The effect of volume status on erythropoietin production in elite athletes has been described (58). In contrast to the inverse relationship of hematocrit and serum erythropoietin in non-renal anemia, subjects who were volume expanded had both lower hematocrit and lower serum erythropoietin levels. Fractional sodium reabsorption was the strongest predictor of the change in serum erythropoietin (58). Further, human subjects, who underwent volume contraction by plasmapheresis, developed higher hemoglobin and higher erythropoietin levels (60). These examples of the paradoxical relationship between erythropoietin and hematocrit in these healthy subjects suggests that the standard relationship of the hemoglobin and serum erythropoietin is modulated by the ECF volume status, possibly through the renin-angiotensin system (Figure 3C)

The hematological adaptation requires intense exercise accompanied by exercise induced hypoxia (59). The response can be further modulated by changes in ECF volume as suggested by the differential response to similar hypoxic exposures during exercise and at rest (60). Parallel effects are seen during the hematological adaptation at altitude (28) and are likely mediated by changes in PO_2.

The production of erythropoietin by the kidney is likely modulated by both the delivery of oxygen and the renal tubular work and O_2 consumption. The hematocrit that is established in light of the ECF & RBC adaptations to exercise likely represents the optimal hematocrit for the physiological parameters and hence differs amongst the types and intensity of exercise.

SUMMARY

The concept of a *critmeter* within the kidney establishes a role of the kidney not only in regulating ECF volume and RBC mass, but in integrating these two volumes to generate the hematocrit. As the 'normal' hematocrit is not a random number, but one that maximizes tissue oxygen delivery, it follows that the hematocrit should be regulated. It is hypothesized that the *critmeter* is a functional unit established by nephron heterogeneity in renal blood flow and in the reabsorption of sodium under physiological conditions. The kidney may uniquely translate a measure of plasma volume into a tissue oxygen pressure signal by the effects of sodium reabsorption on renal energy utilization and oxygen consumption. The RBC mass and plasma volumes are likely integrated at the level of the tissue partial oxygen pressure by the balance of oxygen consumption required for sodium reabsorption and oxygen delivery to the proximal tubule. This balance may be modulated by the renin-angiotensin system in that Ang II affects these variables disproportionately. Clinical and experimental evidence supports the interaction of the renin-angiotensin system with the production of erythropoietin. In terms of blood volume regulation, the effects of the renin-angiotensin system on erythropoietin production allows for the efferent signal of volume regulation to more closely reflect the components of the afferent signals (Figure 4). Poposed examples of resetting or dysfunction of the *critmeter* are the functional deficiency of erythropoietin in some forms of chronic renal failure and in sports anemia.

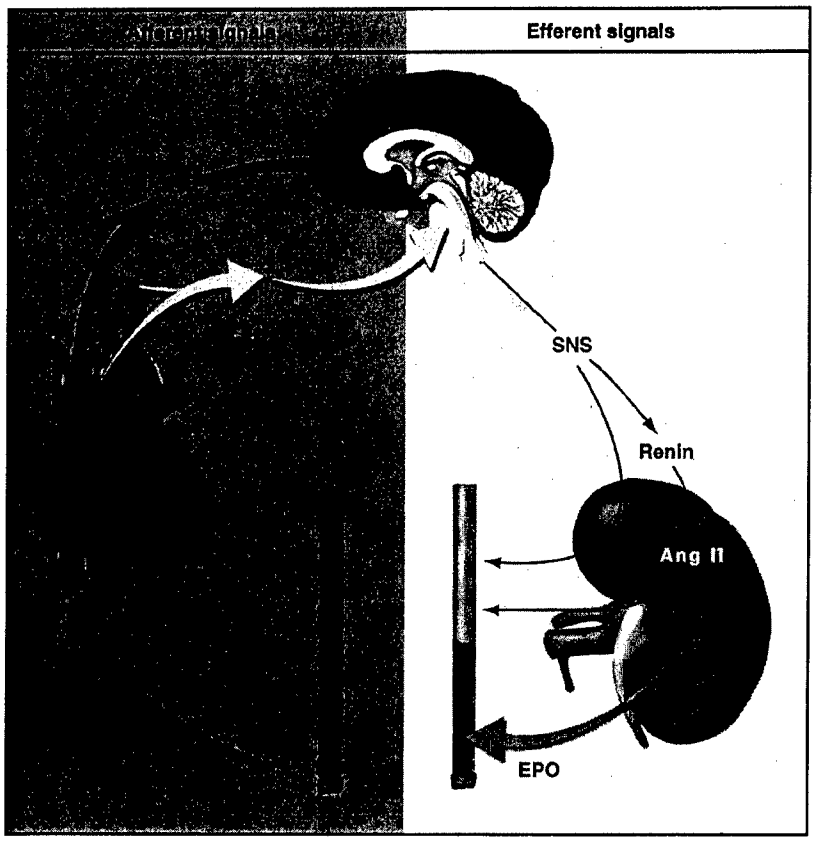

Figure 4. Whereas afferent signals speak to total blood volume that consists of both the RBC mass and the plasma volume, the efferent signals, as classically described, regulate the plasma volume only. Incorporating the production of erythropoietin within this feedback loop provides a more comprehensive integration of the components of blood volume and its regulation. (Used with permission from Elsevier: Reprinted from American Journal Of Kidney Disease, V38(2), Donnelly S, "Why is Erythropoietin Made in the Kidney? The Kidney Functions as a Critmeter", 415-425, 2001.

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Chapter 7

HYPOXIA AND HIGH ALTITUDE

The molecular response

Gisele Höpfl, Omolara Ogunshola, Max Gassmann

Abstract: Increased erythropoietin plasma levels and the consequent augmented production of red blood cells is the best known systemic adaptation to reduced oxygen partial pressure (pO_2). Intensive research during the last years revealed that the molecular mechanism behind the regulation of erythropoietin is ubiquitous and has far more implications than first thought. Erythropoietin regulation results from the activation of the hypoxia-inducible factor-1 (HIF-1) pathway under hypoxic conditions. HIF-1 is a heterodimer consisting of an oxygen sensitive - HIF-1α - and an oxygen-independent subunit - HIF-1β (also known as the aryl hydrocarbon receptor nuclear translocator - ARNT). In addition to erythropoietin, more than 30 genes are now known to be up-regulated by HIF-1. Recently, the critical involvement of HIF-1α post-translational modifications in the cellular oxygen sensing mechanism was discovered. In this review we will focus on the regulation of the HIF-1 pathway and the cellular oxygen sensor and discuss their implications in high altitude hypoxia.

Key Words: HIF-1, oxygen sensing, erythropoietin, VEGF

INTRODUCTION

The use of oxygen as an electron acceptor in the respiratory chain and the consequent higher efficiency in the production of chemical energy (adenosine 5'-triphosphate - ATP) has allowed the development of higher, multicellular forms of life. The essential energetic role of oxygen makes maintenance of oxygen homeostasis a critical survival issue. Mechanisms to sustain this homeostasis, for instance by sensing and regulating oxygen levels, are found throughout the evolutionary tree. Hypoxia is a state where oxygen availability/ delivery is below the level of necessary to maintain physiological O_2 tensions of a particular tissue i.e. when the tissue demand exceeds its O_2 supply. In other words, hypoxia results from an imbalance between demand and supply of O_2. It is of note that different

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tissues have different oxygenation levels already at sea levels. For instance, a mean pO_2 of 18 mmHg (2.5%) was measured at 1mm depth in the cerebral cortex (159) whereas in the renal cortex 20-30 mmHg of oxygen (3-4%) have been measured (33, 130). Although these tissues appear hypoxic, this is their normal physiological O_2 levels at which homeostasis is maintained.

Hypoxia can occur at both local and systemic levels. Systemic hypoxia in mammals occurs mostly in high altitude, in case of congenital or acquired heart or lung disease or in anemia whereas local (or tissue) hypoxia results in most cases from impaired/insufficient vascular supply as for example in stroke, coronary insufficiency or solid tumors. Systemic hypoxic exposure triggers two main responses: a systemic (organism) response and a cellular one. The systemic response is mediated by chemoreceptors and the central and peripheral nervous system causing changes in overall physiological parameters such as respiration and heart rate. The cellular response is mediated by the hypoxia-inducible factors (HIFs). The best studied factor of this group is the hypoxia inducible factor 1 (HIF-1), a heterodimer consisting of the subunits HIF-1α and HIF-1β. HIF-1α protein is not detected under normoxic conditions but increase exponentially with decreasing pO_2 whereas HIF-1β is not affected by oxygenation levels. Activation of the HIF-1 pathway has consequences for the cellular as well as systemic adaptation to hypoxia. The activation of the HIF-1 pathway, its regulation and its importance for the molecular response to hypoxia are going to be discussed in this review.

HIF-1 PATHWAY: AN OVERVIEW

HIF-1α is the most important protein regulating the mammalian molecular response to hypoxia. Hypoxia may have a rapid onset and be potentially deleterious or even lead to cell death in case actions are not immediately taken to counteract or neutralize its effects. In order to achieve an immediate response to this potentially life-threatening state, HIF-1α is regulated at the protein level. This means that HIF-1α protein is being constantly synthesized and constantly degraded already under normoxic conditions (cf. Figure 1). The degradation of HIF-1α is promoted by prolyl-hydroxylases, that require oxygen and 2-oxoglutarate as co-substrates as well as iron and ascorbic acid as co-factors. Hydroxylated HIF-1α binds to the von Hippel Lindau protein (pVHL), a subunit of a multiprotein harbouring E3 ubiquitin-ligase activity. Subsequent ubiquitination primes HIF-1α for proteasomal degradation. The current idea is that under reduced pO_2 (hypoxia), less oxygen is available for the hydroxylation reaction that in turn results in stabilization of HIF-1α. Non-hydroxylated HIF-1α is phosphorylated by several kinase pathways, translocates to the nucleus where it dimerizes with its partner HIF-1β and binds as a dimer - now called HIF-1 - to hypoxia response elements (HREs) found in promoter/enhancer elements of several genes involved in improving oxygenation, energy production and cell survival through a number of different mechanisms. The recruitment of coactivators such as CBP (CREB-binding protein; CREB is the abbreviation for the cyclic-AMP response element)/p300, is essential for the transcriptional up-regulation of these genes (4, 29). Also phosphorylation of HIF-1α (which mostly occurs through growth factor stimulation) is important for the transactivation of HIF-1 target genes. Another important factor leading to the accumulation of HIF-1α protein is the loss of pVHL, a protein involved in HIF-1α degradation (90).

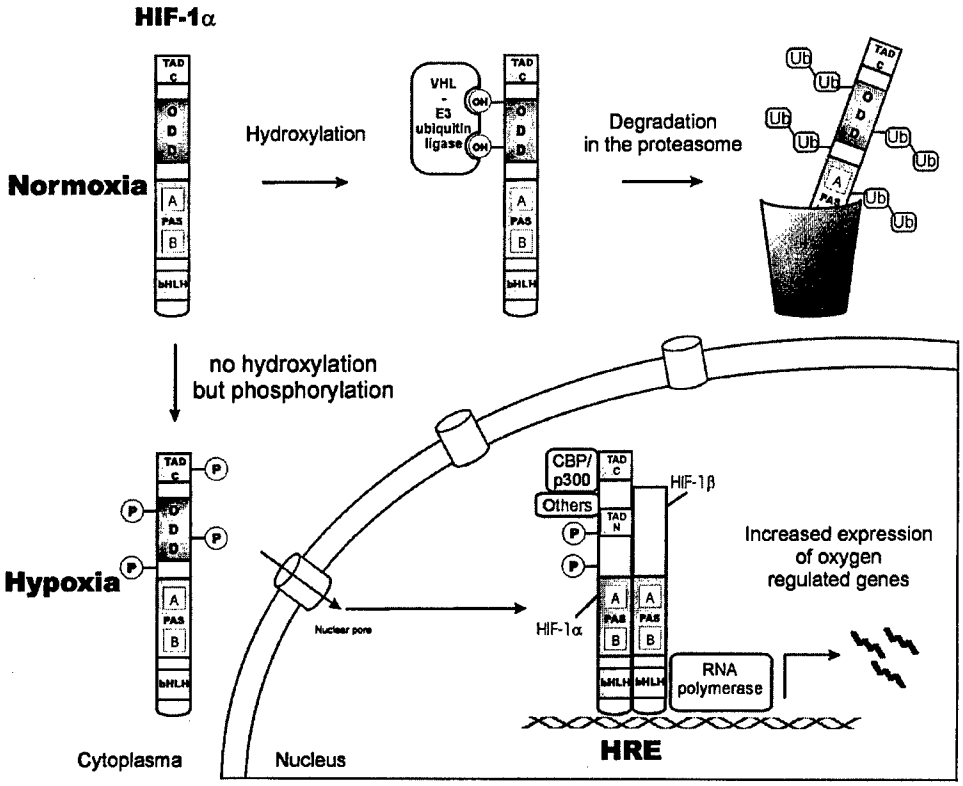

Figure 1. HIF-1 Pathway: an overview - Under normoxic conditions, HIF-1α is prolyl-hydroxylated and targeted for degradation in the proteasome. Under hypoxic conditions it is phosphorylated, translocates to the nucleus and dimerizes with its partner HIF-1β (ARNT) forming HIF-1. After co-factor recruitment, HIF-1 up-regulates the transcription of oxygen regulated genes such as erythropoietin. VHL: von Hippel Lindau protein (for details see text).

HYPOXIA-INDUCIBLE FACTOR-1

The erythropoietin-regulated increase in hematocrit is a long known adaptation mechanism to exposure to high altitude hypoxia in humans. In 1995, Semenza and Wang took the first step towards decoding of the mechanism behind the hypoxic regulation of erythropoietin by cloning a factor interacting with the 3' region of the erythropoietin gene (153). As this factor only bound after hypoxic exposure it received the name "hypoxia-inducible factor-1" (155). Further purification revealed that HIF-1 consists of two subunits termed HIF-1α and HIF-1β, respectively (153). Microsequencing of these polypeptides showed that HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT), a protein already known to be constitutively expressed. HIF-1α was a novel, highly conserved protein of 826 amino acids (153). The availability of antibodies against recombinant HIF-1α and HIF-1β peptides (13, 153) allowed the characterization of the expression of these proteins in cultured cells as a function of the oxygen concentration. No HIF-1α protein was de-

tected in cells cultured under normoxic conditions (21% O_2). However, hypoxic exposure (1% O_2) resulted in stabilization/accumulation of HIF-1α protein and therefore allowed its detection. Using tonometers, Jewell *et al.* (77) detected HIF-1α protein in the nucleus after less than 2 minutes of exposure to hypoxia/anoxia. Further experiments using HeLa cells showed that the levels of expression of HIF-1α protein and DNA-binding of HIF-1 vary exponentially over a physiologically relevant range of O_2 tension increasing exponentially as O_2 concentration declines (80). Reoxygenation reduced HIF-1 DNA binding and nuclear HIF-1α protein levels within 4 to 8 minutes suggesting a protein half-life of approximately 5 minutes (77). In short, under hypoxic conditions, HIF-1α protein is stabilized, translocates into the nucleus and accumulates within a very short period of time (minutes) to allow rapid responses to lowered oxygen concentrations. When oxygen levels rise, HIF-1α is immediately degraded so that it is undetectable under normoxic conditions.

HIF-1α Structure

HIF-1α and HIF-1β share two structural characteristics. They both contain basic helix-loop-helix (bHLH) and PAS domains (PAS is an acronym referring to the first proteins - PER, ARNT, SIM - in which this motif was first identified - cf. Figure 2). The basic domain and the C-terminal half of PAS are specifically required for DNA binding while the HLH domain and the N-terminal half of the PAS domains are required for the formation of the HIF-1α/HIF-1β heterodimer capable of binding to DNA (79). DNA sequences necessary for the binding of HIF-1 are termed "hypoxia response elements" or HREs. Binding of HIF-1α to the HREs leads to the up-regulation of HIF-1 target genes. New studies demonstrate that not only the amount of protein but also further factors such as coactivators or protein modifications determine the binding of HIF-1 to HREs and are important for the activation of target genes (cf. Figure 1). For instance, an overexpression study (61) showed that *in vitro* 100-fold overexpressed HIF-1α translocates into the nucleus under normoxic and hypoxic conditions but is neither able to further increase the HIF-1 binding capacity nor the mRNA levels of HIF-1 target genes beyond the levels found in control cells exposed to hypoxia.

HIF-1α also contains two transactivation domains (TADs). The main function of the TADs is to recruit and interact with coactivators that will lead to the transcriptional activation of target genes. These domains are important because HIF-1α undergoes post-translational regulation, being mediated through hydroxylation, phosphorylation, acetylation and/or redox modifications of these two TAD domains (11, 67, 76, 118). Cells cultured under hypoxic conditions (typically 1% O_2) increase HIF-1α protein levels without concomitant elevation in mRNA expression. This suggests that the main regulation pathways rely on oxygen-dependent protein stabilization (48, 67). Amino acids 401-603 of HIF-1α comprise a sequence that is both necessary and sufficient for regulation of protein stability as a function of the O_2 concentration (68) and is therefore called oxygen-dependent degradation domain or ODD-domain (cf. Figure 2).

Upon stabilization, HIF-1α accumulates in the nucleus of hypoxic cells (22, 78). Two nuclear localization signals have been identified: aas 17-33 (NLS-N) within the bHLH domain and aas 718-721 (NLS-C) within the C-terminal regulatory domain (81) – (cf. Figure 2). Hypoxic HIF-1α translocation and nuclear accumulation still occur in HIF-1β/ARNT-deficient hypoxic embryonic stem cells demonstrating that these events are ARNT-

independent. This result was substantiated by the finding that HIF-1β/ARNT is a nuclear protein (22).

In summary, HIF-1α protein belongs to the bHLH/PAS superfamily. It has a defined domain architecture in which the N-terminal domains are involved in dimerization/DNA binding whereas the C-terminal domains are concerned with stabilization and transactivation regulation of the heterodimer.

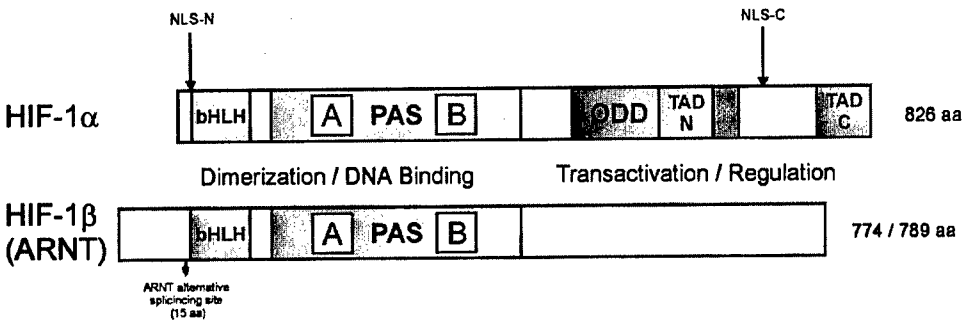

Figure 2. Structural features of HIF-1α and HIF-1β/ARNT: Basic helix-loop-helix (bHLH) and PAS domains of both subunits are shown together with amino- (N) and carboxyl- (C) terminal nuclear localization signals (NLS), N and C terminal transactivation domains (TAD-N and TAD-C) and the oxygen-dependent degradation (ODD) domain. The number of amino acids of each protein is indicated. The alternative splicing site of ARNT is also shown.

HIF-1α and ARNT Sequence Homologues

Two other proteins belonging to the bHLH-PAS superfamily and showing striking homology with HIF-1α have been discovered. These proteins share several characteristics with HIF-1α, such as hypoxic protein stabilization, heterodimerization with ARNT(s), DNA-recognition/binding and reporter gene transactivation. The best studied protein was first termed endothelial PAS protein (EPAS-1) (148). Wenger and Gassmann (158) proposed this novel protein to be named "HIF-2α" because of its similar domain architecture to HIF-1α (cf. Figure 3). Recent studies (160) showed that HIF-2α is expressed in a complementary but not overlapping pattern to HIF-1α in specific cells of most organs after systemic hypoxic exposure. A third protein, called HIF-3α also shares considerable sequence homology with HIF-1α and HIF-2α (49) but lacks a C-terminal transactivation domain (TAD-C). This characteristic accounts for its inhibitory effects over HIF-mediated transcription (56). The expression of HIF-3α was found in the distal tubules of the kidney and led to the suggestion that it could act as a negative regulator of the HIF pathway in this tissue (56). In accordance with this suppression effect a dominant negative regulator of the HIF-αs (the inhibitory PAS domain protein - IPAS) was identified as a splice variant of the HIF-3α locus (101). This protein is expressed in Purkinje cells and in the corneal epithelium of the eye, where it is thought to play a role in maintenance of the avascular phenotype of this tissue by forming non-functional complexes with the HIFs (100).

As HIF-1α, sequence homologues of HIF-1β (ARNT) have also been discovered in recent years and may play physiological roles as β-class partners of the HIF-α subunits. The ARNT2 protein (28, 59) has been shown to substitute ARNT in heterodimerizing with HIF-1α, HIF-2α and HIF-3α prior to HRE binding in DNA-binding assays (59). Furthermore, forced expression of ARNT2 in ARNT - deficient cells rescued hypoxic gene induction (103) and partial redundancy with ARNT has also been shown *in vivo* (84). A striking difference between these proteins is that the pattern of expression of ARNT2 is restricted primarily to brain and kidney (28, 59, 75), whereas HIF-1β is ubiquituously expressed. In contrast to ARNT2, a third ARNT homologue, MOP3 (member of PAS 3, also called BMAL1- brain and muscle ARNT-like protein 1 or simply ARNT3) (63 , 70, 145) is a weak dimerization partner of the HIF-α(s) (64, 75) and was shown not to participate in the hypoxic response (26). The multiple possibilities given by the amount of dimerization partners and diverse expression patterns add to the complexity of the hypoxic signaling response and the precise role of these molecules still remains to be elucidated.

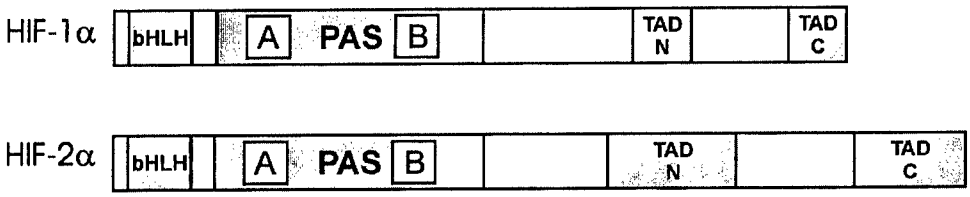

Figure 3. HIF-1α and HIF-2α proteins have similar structural domains: Both proteins show a high degree of homology with similar structural features including basic helix loop helix (bHLH), PAS and - N and C - terminal transactivation domains (TAD-N and TAD- C).

HIF-1α Phosphorylation – Regulation of HIF-1 by External Stimuli

The first studies showing the fundamental characteristic of HIF-1α protein stabilization under hypoxic conditions were seminal for the understanding of the HIF-1 mediated hypoxic response. Further studies (141) have shown that HIF-1α protein can be stabilized already under normoxic conditions in physiological as well as pathological situations (141). Although little is known about mechanisms regulating these processes, post-translational modifications such as phosphorylation seem to play a major role in the HIF-1α activation under both normoxic and hypoxic conditions.

There are two main phosphorylation cascades involved in HIF-1α activation. They can be initiated after growth factor receptor binding to receptor tyrosine kinases, which in turn activate the downstream targets. This is of importance because different levels of growth factors and their receptors can modulate/activate the HIF pathway through stimulation of phosphorylation cascades. The three broad subfamilies of the mitogen-activated protein kinase (MAPK) cascades, namely the c-Jun NH_2-terminal kinases (JNKs), p38 MAPKs and the extracellular signal-regulated kinases (Erks) have been shown to be involved in the regulation of the HIF-pathway (2, 41, 109, 118, 135). Interestingly, although HIF-1α is a highly phosphorylated protein and two Erk1/Erk2 MAPK consensus sites (PXSP) ex-

ist on the human HIF-1α (positions 515 and 687), point mutations did not reveal the exact phosphorylation sites (118).

The best studied MAPK pathway involved in HIF-1α regulation is the one leading to the activation of Erk1-2 (also called p44/42) after the activation of the downstream molecules Ras/Raf-1/MEK-1/Erk1-2 (for MEK pathway review (54)) (cf. Figure 4). An indication that HIF-1α phosphorylation can be mediated by growth factor binding was achieved using the cell-impermeable organomercurial compound mersalyl (1). The effects of mersalyl (which binds to the IGF-1 receptor-IGF-1R) on HIF-1α could only be blocked by the MEK-1 inhibitor PD098059, but not by the phosphatidylinositol-3- kinase inhibitor wortmannin. Hur *et al.* (69), who used MEK-1 inhibitors such as PD098059, suggested that this pathway is only involved in transactivation but does not seem to influence the stabilization or DNA-binding ability of HIF-1α. This finding was confirmed by another group (118). Furthermore, in HIF-1α-overexpressing HeLa cells PD098059 completely abolished *trans*-activation activity of both normoxic and hypoxic overexpressed HIF-1α without compromising stabilization (61) demonstrating the importance of the MAPK pathway for the functionality of HIF-1α.

The two stress activated serine/threonine protein kinases JNK and p38 MAPK have also been shown to increase activity under hypoxic conditions in certain cell lines (24, 131, 133). For instance, p38 MAPK activation was shown to be involved in the activation of the HIF pathway in vascular smooth muscle cells (VSMC) by thrombin (47) and in prostate carcinoma cells by Cr(IV) (41).

Another important HIF-1α phosphorylation pathway is the phosphatidylinositol (PI) -3-kinase (PI3K) signaling cascade. PI3K catalyses the conversion of PI-4-phosphate and PI-4,5-biphosphate to PI-3,4-biphosphate and PI-3,4,5-triphosphate respectively, which are allosteric activators of PI-dependent kinase-1 (PDK-1) and of the Akt kinase (also known as protein kinase B). Allosteric activation of PDK-1 leads to phosphorylation and activation of Akt. Akt has several downstream targets involved in apoptosis, cell cycle and growth as well as translation (150). One of these targets is FRAP (FKBP12/rapamycin-associated protein, also known as mTOR - mammalian target of rapamycin – cf. Figure 4). FRAP is in turn an activator of p70 ribosomal protein S6 kinase ($p70^{s6k}$) (9, 128, 167), a kinase that enhances the translation of mRNAs that have 5' polypyrimidine tracts as it can be found in HIF-1α (73). In addition, it phosphorylates the translational regulatory protein 4E-binding protein (4E-BP). Hyperphosphorylation of 4E-BPs leads to the release and activation of the eukaryotic translation initiation factor 4E (eIF-4E) which binds together with other factors to 5' mRNA cap structure and allows the recruitment of ribosomes increasing the translation rate (44, 117). Both FRAP and $p70^{s6k}$ are inhibited by rapamycin (8). This pathway is also negatively regulated by PTEN (phosphatase and tensin homologue deleted on chromosome 10), which dephosphorylates PI-3,4-bisphosphate and PI-3,4,5-trisphosphate (for review see (14, 150)).

Despite growing evidence about the involvement of the PI3K pathway in the regulation of HIF-1α (especially in cancer (40, 95, 167)), two new reports challenge this notion (3, 5). In several different cell lines, chemical or genetic inhibition of PI3K as well as activation of the PI3K/Akt pathway with growth factors/overexpression of active mutants of PI3K or Akt had only modest effects in HIF-1α protein stabilization or activity. They propose that the activation of this pathway is not sufficient for HIF-1α induction and is not essential for its regulation by hypoxia.

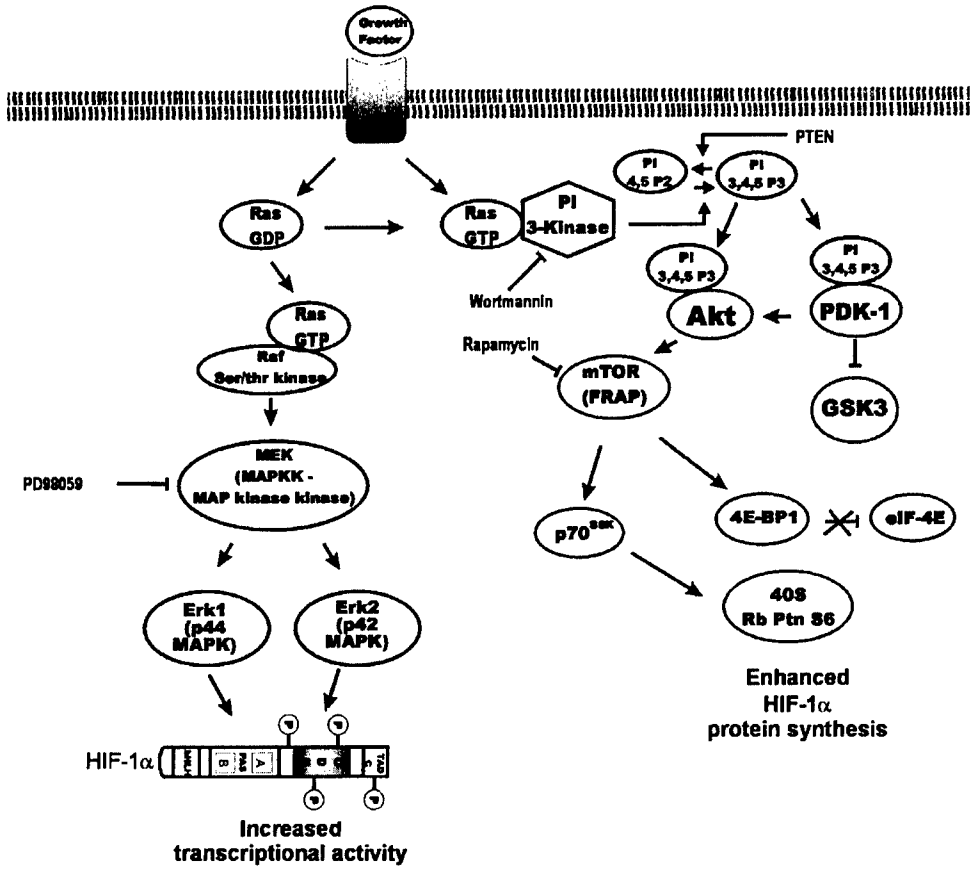

Figure 4. Phosphorylation pathways induce increased HIF-1α expression and activity: The two main pathways leading to phosphorylation of HIF-1α. Abbreviations are: Erk: extra cellular signal-related protein kinase, GSK3: glycogen synthase kinase-3, PDK1: phosphatidylinositol-dependent protein kinase-1, MAPK: mitogen-activated protein kinase, MEK: MAP-ERK-activating kinase, MEKK: MEK-kinase, mTOR: mammalian target of rapamycin PI: phosphatidylinositol, PTEN: Phosphatase and tensin homologue deleted on chromosome 10, $p70^{S6K}$: p70 ribosomal protein S6 kinase, 4E-BP: 4E-binding protein, eIF-4E: eukaryotic translation initiation factor 4E, 40S Rb Ptn S6: 40S ribosomal protein S6

In summary, HIF-1α phosphorylation involves may different complex signaling cascades. It can be visualized via a change in the eletrophoretic migration pattern of extracts from cells exposed between 10 and 30 minutes to hypoxia/anoxia (77) and is important for the activity of HIF-1 since overexpressed HIF-1α is stable under normoxic conditions but not fully active (61). The exact contribution of each phosphorylation pathway to the HIF-1α induction of expression, stabilization, DNA-binding and activation of target genes still remains obscure.

Growth Factors and Others –Shaping HIF-1-Mediated Responses

The first two peptide mediators identified to activate the HIF-1 pathway were insulin and insulin-like growth factor-1 (IGF-1) (166). The hint leading to this discovery was the fact that insulin, IGF-1 and HIF-1 share the ability to induce the expression of a similar set of genes such as *Glut-1, Epo*, and *VEGF* (105, 123, 156). Zelzer *et al.* (166) demonstrated that insulin and IGF-1 not only induce the *in vitro* formation of a transcriptionally active HIF-1-complex, but are also able to stabilize this complex. Other growth factors capable of inducing HIF-1α expression in cultured cells are insulin-like growth factor-2 (IGF-2) (34) and epidermal growth factor (EGF) (167).

A link between HIF-1α and inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) has also been shown. Both stimulate DNA-binding of HIF-1 and increase HIF-1α protein expression (58, 140). Interestingly, whereas the IL-1 effect is mediated by the PI3K pathway (140), TNF-α seems to modulate HIF-1α activity through a reactive oxygen species (ROS) sensitive pathway (51, 126). In addition, gingival and synovial fibroblasts incubated with IL-1 increased HIF-1α mRNA levels (147).

Several factors, such as angiotensin II, thrombin, and platelet-derived growth factors mediate normoxic induction of HIF-1α in vascular smooth muscle cells (47, 119). These effects are abolished by addition of antioxidants, suggesting the participation of ROS in the transduction pathway. The importance of redox processes in HIF-1 response has come to light in recent years with reports revealing that ROS may trigger HIF-1α function (16, 31, 52, 53, 67, 94, 125, 154). However, the role of the mitochondrial respiratory chain - one of the most well known sources of ROS - is still controversial (18, 19, 138, 149).

Many growth factors can influence and modulate the HIF response even leading to its activation already under normoxic conditions. This modulation results mostly from post-translational modifications of the HIF-1α protein, most especially phosphorylation. This is of importance because the pleiotropic effects resulting from the activation of this pathway can, in case of necessity, be used for cell survival even in the absence of hypoxia.

HIF-1: The Target Genes

The central importance of HIF-1 in the hypoxic response and the involvement of hypoxia in a variety of pathological diseases brought about a great deal of research in this field in recent years. To date, over three dozen HIF-1 target genes have been identified (cf. Table 1) and the list grows steadily. Considering this aspect, the list below does not intend to be complete but representative for these genes.

Most of the target genes up-regulated by HIF-1α activation improve cell or systemic survival. The best examples are the genes involved in erythropoiesis and vascular control, which will improve the delivery of oxygen to tissues either through increased blood oxygen carrying capacity or increased blood vessel density/dilatation. Increased expression of glycolytic enzymes and glucose transporters, improving the cellular energy gain through anaerobic glycolysis, is also characteristic for the hypoxic response.

Table 1. HIF-1 Target Genes

Gene Product	Function	References
α_{1B}-adrenergic receptor	Vascular tone	(30)
Adenylate cyclase	Nucleotide metabolism	(161)
Adrenomedullin	Vascular tone, cell survival	(25, 42)
Aldolase A	Glucose metabolism	(72, 124)
Aldolase C	Glucose metabolism	(72)
Carbonic anhydrase 9	pH regulation	(162)
Ceruloplasmin	Iron metabolism	(111)
Endothelin-1	Vascular tone	(12, 66)
Enolase 1	Glucose metabolism	(72)
Erythropoietin	Erythropoiesis, cell survival	(79)
Glucose transporter 1	Glucose metabolism	(72, 124, 161)
Glucose transporter 3	Glucose metabolism	(64)
Glyceraldehyde-3-P-dehydrogenase	Glucose metabolism	(72, 124)
Heme oxygenase	Vascular tone, cell survival	(96)
Hexokinase 1	Glucose metabolism	(72)
Hexokinase 2	Glucose metabolism	(72)
IGF-binding protein 1	Cell proliferation and survival	(146)
IGF-binding protein 2	Cell proliferation and survival	(34)
IGF-binding protein 3	Cell proliferation and survival	(34)
Insulin-like growth factor (IGF-2)	Cell proliferation and survival	(34)
Lactate dehydrogenase A	Glucose metabolism	(72, 124)
NIP3	Apoptosis	(10)
Nitric oxide synthase 2	Vascular tone, cell survival	(115)
p21	Cell proliferation	(15)
p35srj	Regulation of HIF-1 activity	(6)
Phosphofructokinase L	Glucose metabolism	(72)
6-Phosphofructokinase-2-kinase/ fructose –2,6-biphosphatase	Glucose metabolism	(108)
Phosphoglycerate kinase 1	Glucose metabolism	(15, 72, 124)
Plasminogen activator inhibitor 1	Angiogenesis	(86)
Prolyl-4-hydroxylase α(I)	Collagen metabolism	(144)
Pyruvate kinase M	Glucose metabolism	(72)
RTP801	Apoptosis/cell survival	(137)
Transferrin	Iron metabolism	(121)
Transferrin receptor	Iron metabolism	(99)
Transforming growth factor β3	Angiogenesis, cell proliferation	(143)
Triosephosphate isomerase	Glucose metabolism	(72)
Vascular endothelial growth factor	Angiogenesis, cell survival	(15, 72, 124)
VEGF receptor 1 (flt-1)	Angiogenesis	(43)

As previously mentioned, one of the most prominent systemic responses to hypoxia is the up-regulation of the peptide hormone erythropoietin that ultimately leads to increased blood oxygen carrying capacity. In order to produce more erythrocytes capable of transporting oxygen the bone marrow also needs to produce more heme, and so the iron demand

will increase concomitantly with the erythrocyte production. Since iron availability is the most common limiting factor in erythropoiesis, HIF-1 up-regulates a series of genes that facilitate the delivery of iron to erythroid tissues. As such transferrin, a blood iron transporting molecule, as well as its receptor are up-regulated under hypoxic conditions (99, 121, 143). Ceruloplasmin, another HIF-1 target gene, is a ferroxidase required to oxidize ferrous to ferric iron (the only form of iron binding to transferrin). Its up-regulation is also likely to improve iron delivery to erythroid tissues (111). Thus, hypoxia and HIF-1 not only lead to an increase in erythrocyte number, but also control many of the factors necessary for this process to take place. It should be noted that processes related to heme synthesis can also be up-regulated by hypoxia independent of HIF-1. An example is the HIF-1-independent regulation of erythroid-specific 5-aminolevulinat synthase (ALAS2), the rate limiting enzyme of the heme pathway in erythroid tissues (62).

Furthermore, proteins involved in the formation, remodeling flexibility and tone of the vascular system are also regulated by HIF-1. The most eminent molecule of this group is the vascular endothelial growth factor (VEGF) due to its involvement in a series of important human pathologies characterized by hypoxic states such as ischemia/reperfusion, stroke or cancer (38, 97, 98). VEGF is a potent promoter of angiogenesis, a process in which new capillaries spread from pre-existing ones, and is thought to improve tissue oxygenation through capillary density increase (35). It is also the only molecule known to be heterozygote lethal (36), demonstrating its fundamental importance in embryonic development. A number of molecules controlling vascular tone are also regulated by HIF-1. Examples are the up-regulation of the enzymes inducible nitric oxide synthase and heme oxygenase. Their up-regulation will in turn increase the production of NO and CO respectively, causing vasodilation (96, 106, 115). Other hypoxia-mediated effects on vascular tone are mediated by adrenomedullin, which is a hypotensive peptide expressed at the mRNA level in adult ventricular cardiac muscle (25, 42, 112). Interestingly, the vasoconstrictor endothelin-1 also seems to be hypoxia regulated but its regulation is attenuated in comparison with other hypoxia regulated genes (12, 66).

Another hallmark of the hypoxic response is the up-regulation of oxygen-independent metabolic pathways to enhance the production of energy under reduced pO_2. Energy production under normoxic conditions relies mostly on the mitochondrial respiratory chain in which oxygen is the final electron acceptor. Thus, reduced pO_2 leads to decreased energy production under hypoxic conditions (45). In an attempt to compensate for this energy decline, glycolysis must be stimulated where ATP production occurs much faster but inefficiently. This fact was first seen in the pioneering work of Louis Pasteur who observed that yeast growing under anaerobic conditions needed considerably larger quantities of sugar to produce energy. This is not only true for yeast, but also for almost any other type of cell and is now known as the Pasteur Effect. HIF-1α has been shown to be essential for this effect to occur (132). With this in mind it is unsurprising that almost every enzyme involved in the glycolytic pathway, as well as glucose transporters that are central for glucose uptake, are up-regulated by HIF-1 (cf. Figure 5) (72, 157). In order to assure a high glycolytic flux under hypoxic conditions, the rate-limiting enzyme of the glycolytic pathway, namely phosphofructokinase (PFK), is activated allosterically by the potent regulator fructose-2,6-bisphosphate (F-2,6-P2). Its synthesis and degradation depends upon the enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB), which has both kinase and phosphatase activities. One of the isoforms of this enzyme, PFKFB3, has the highest

kinase/phosphatase ratio and therefore is able to increase the levels of F-2,6-P2. PFKFB3 is induced at the mRNA level by hypoxia in a HIF-1-dependent manner (108). This means that hypoxic exposure not only increases the expression of almost all enzymes of the glycolytic pathway and glucose uptake but also enhances the glycolytic flux by allosteric activation of the rate limiting enzyme of the pathway. Another important factor to consider, apart from the energetic point of view, is that precursors of the pyrimidine/purine pathways are produced during glycolysis. And so stimulation of the glycolytic pathway, for instance in hypoxic cancer cells, may indirectly facilitate proliferation by enhancing the supply of DNA-precursors.

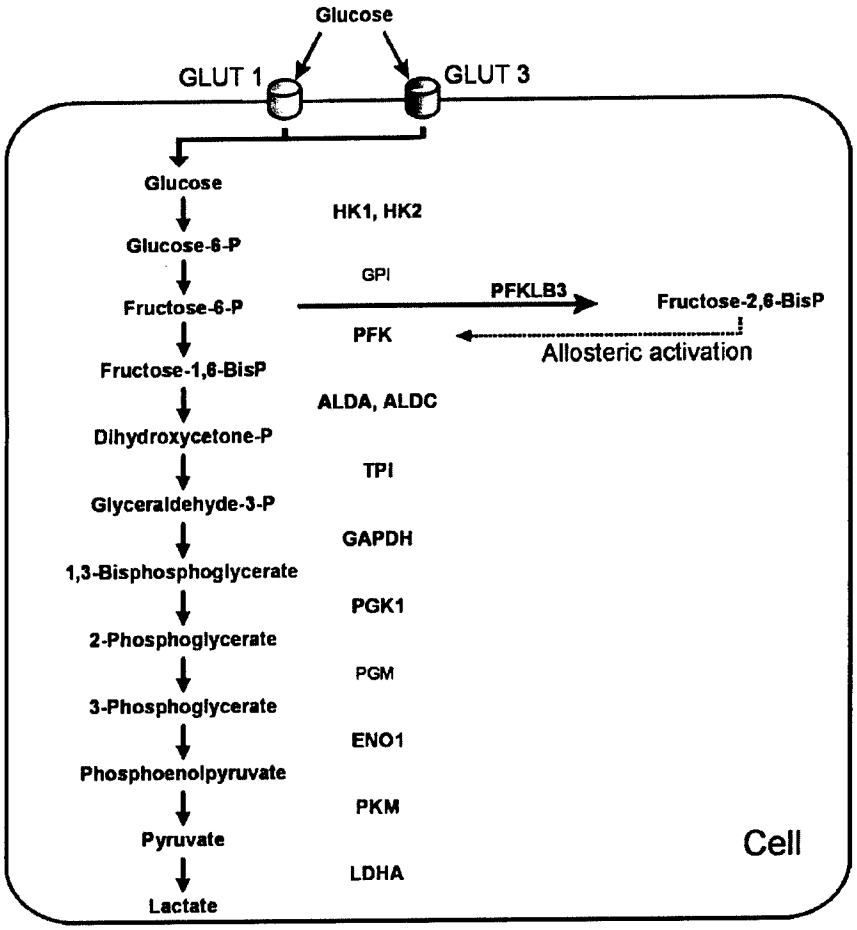

Figure 5. Enzymes of the glycolytic pathway up regulated by HIF-1α: Enzymes represented in bold are up-regulated by HIF-1 (72, 161). GLUT1 and GLUT3: glucose transporters; HK1 and HK2: hexokinase 1 and 2; GPI: glucosephosphate isomerase; PFKL: phosphofructokinase L, ALDA and ALDC. Aldolase A and C; TPI: triosephosphate isomerase; GAPDH: glyceraldehydephosphate dehydrogenase; PGK1: phosphoglycerate kinase 1; PGM: phophoglucomutase; ENO1: enolase; PKM: piruvate kinase M; LDHA: lactate dehydrogenase A; PFKLB3: 6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3.

Considering the large number of genes up-regulated by HIF-1 and involved in cell survival, it seems contradictory that it should also up-regulate genes involved in programmed cell death. Nevertheless, the pro-apoptotic protein Nip3 (21) has been shown to be up regulated by prolonged exposure to hypoxia (only expressed after 4 days of exposure to 0.5% O_2) (10). Promoter analysis of Nip3 revealed two putative HREs. Luciferase reporter assays using this promoter in cells transfected with HIF-1 showed activation under normoxic conditions (10). Furthermore, mutation of either HRE leads to abolishment of the signal demonstrating that the Nip3 promoter is HIF-1 responsive. The authors speculate that, due to the slow response and modest apoptotic activity, hypoxic cells/tissues would have a critical opportunity to adapt to oxygen deprivation by means of activation of an initial HIF-1-dependent protective response. However, persistent O_2 deprivation might cause Nip3 accumulation and promote cell death.

In summary, hypoxic stimuli are crucial for the regulation of tissue homeostasis and adaptation of several systems to low oxygen conditions. By up-regulating genes that improve metabolic status and at the same time improve oxygen delivery to the tissue (by increased number of erythrocytes and/or increased capillary density) HIF-1 exerts pleitropic effects that considerably improve cell survival under hypoxia.

OXYGEN SENSORS

The nature of the oxygen sensor remained unknown for many years and gave rise to a series of theories discussed elsewhere (157). The breakthrough in this field was achieved by two groups simultaneously (71, 74). They discovered an oxygen-dependent enzymatic modification of the ODD domain of HIF-1α. A newly discovered prolyl-4-hydroxylase is able to hydroxylate two prolin residues (Pro^{402} and Pro^{564}, cf. Figure 6) in the presence of oxygen, ultimately leading to the degradation of HIF-1α. Three HIF-prolyl hydroxylases (named HPH 1, 2 and 3, respectively) were cloned by Jaakkola *et al.* (74) and Buick *et al.* (11). These enzymes require oxygen and 2-oxoglutarate as co-substrates and contain iron bound to two histidines and one aspartic acid residue. In order to retain iron in its enzymatically active ferrous state, ascorbate is also needed. Hypoxia-mimicking elements such as iron chelators or transition metals such as cobalt also suppress the hydroxylation of the proline residues and thus stabilize HIF-1α. A reduction in HIF-1α hydroxylation under hypoxic conditions provided the final proof of the role of these HPHs as oxygen sensors (32).

The prolyl hydroxylation of HIF-1α is of central importance in its degradation pathway. HIF-1α is constitutively expressed, but the protein levels are not detectable under normoxic conditions (46, 77). The degradation pathway starts with the binding of the von Hippel-Lindau tumor supressor protein (pVHL) to the hydroxylated ODD domain of HIF-1α (71, 74). pVHL is part of the E3 ubiquitin-ligase complex that targets key regulatory proteins for ubiquitin-mediated proteolysis in the proteasome (90). Proteasomal inhibitors or mutation of the activating enzyme E1 stabilize HIF-1α, showing that under normoxic conditions HIF-1α is degraded by ubiquitination and proteasomal degradation (68, 82, 116, 125, 139, 142). The loss or mutation of pVHL *in vivo* stabilizes the HIF-1α protein under normoxic conditions and leads to the VHL hereditary cancer syndrome (23, 83, 91, 114, 165).

A new report (76) identified the acetylation of Lys532 within the ODD domain of HIF-1α by the acetyltransferase ARD1. The authors demonstrate that ARD1 inhibits HIF-1α transcriptional activation, protein stability and stimulates its degradation. They suggest that acetylation is, together with hydroxylation, critical for the proteasomal degradation of HIF-1α because it increases the interaction of HIF-1α with pVHL and consequently the pVHL-mediated ubiquitination.

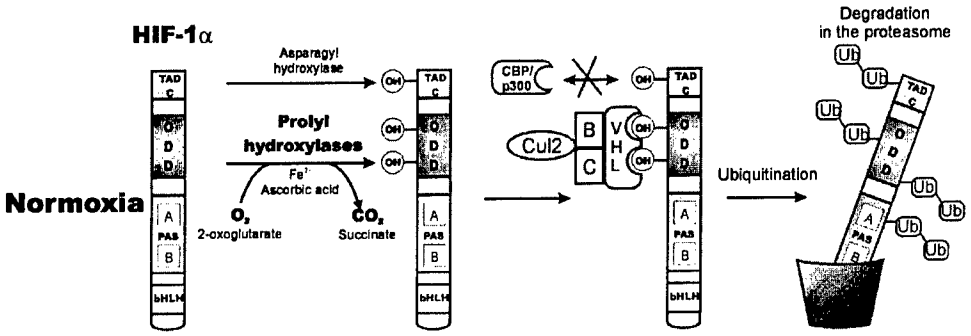

Figure 6. Post-translational modifications lead to degradation of HIF-1α under hypoxic conditions: Both prolyl hydroxylation sites are indicated. The asparagyl hydroxylation site and the binding inhibition of the coactivator CBP/p300 are also represented. pVHL interacts through its β-domain with prolyl-hydroxylated HIF-1α and recruits proteins belonging to the E3 ubiquitin-ligase system such as elongins B and C (B, C) and Cullin 2 (Cul2).

A third hydroxylation site, independent of prolines involved in oxygen sensing, was also determined. Hydroxylation of this site does not lead to HIF-1α degradation and is therefore not involved in the oxygen sensing mechanism. In this case, HIF-1α is hydroxylated in the asparagyl-residue Asn^{803} (located within the C-TAD, cf. Figure 6) and Asn^{851} in HIF-2α (93, 127). The hydroxylation of the Asn^{803} residue leads to a steric inhibition of the interaction between HIF-1α and its coactivator CBP/p300 (27, 39) interfering with its recruitment. This recruitment is critical for HIF-1α activation because initiation of transcription by RNA polymerase II requires sequence specific promoter/enhancer transcription factors as well as basal transcriptional machinery. CBP/p300 may function as a scaffold for the formation of protein complexes or as a bridge for the transcriptional machinery. Aditionally, its histone acetyltransferase activity promotes chromatin remodeling necessary for transcription to occur (for review (17)).

It is of note that the oxygen sensing mechanism described is ubiquitous, since the expression of HIF-1α is unrestricted. Excitable cells, such as glomus cells of the carotid body (sense O_2 in arterial blood) and neuroepithelial cells (O_2 sensing in the lung) also respond uniquely to hypoxia by releasing neurotransmitters. In these cells neurotransmitter release occur after K^+ channel inhibition and voltage-gated Ca^{2+} entry (85). The role of HIF-1α in this process is still under debate and more studies are needed to define the exact role of HIF-1α in these specific cells. However, one report has demonstrated defective carotid body function and impaired ventilatory responses in HIF-$1\alpha^{+/-}$ mice exposed to chronic hypoxia (88).

HIF-1 AND TARGET GENES IN HIGH ALTITUDE

The systemic response to high altitude hypoxia (also called hypobaric hypoxia) depends on intensity and duration of the hypoxic insult. Short exposure times trigger acute responses, long term exposures lead to acclimatization and exposure throughout generations has even given rise to a phenotype found in the native Himalayan and Andean populations (for reviews (60, 110)). The cellular response is also relevant, although it should be noted that carotid body and pulmonary neuroepithelial cells react differently to hypoxia compared to other cells, specifically by the secretion of neurotransmitters. The crucial importance of HIF-1α for cellular oxygen sensing and hypoxic response is well established but not much is yet determined about its effects on hypoxic adaptation/response of tissues or its involvement in the oxygen sensing mechanism in carotid bodies and in neuroepithelial cells. Some of the studies involving HIF-1α and hypobaric hypoxia are presented below.

HIF-1α protein levels, as the physiological response to hypoxia itself, vary with the degree and duration of the hypoxic insult. In addition, each organ has different HIF-1α expression kinetics under hypoxic conditions (141). For instance, in mice exposed to 6% O_2 HIF-1α expression was maximal after 1 to 2 hours in kidney and liver dropping back to normoxic undetectable levels in the third hour of exposure. However, HIF-1α protein could already be detected under normoxic conditions in brain, reaching a peak of expression after 4-6 hours of 6% O_2 exposure (141). The severity of hypoxic exposure necessary to trigger HIF-1α stabilization/up-regulation is also organ specific. While in kidney and liver stabilization was only achieved after systemic exposure to 6% O_2, exposure to 18% O_2 was already sufficient to initiate HIF-1α up-regulation in the brain of mice. The HIF-1α expression in brain during chronic hypoxia (2-3 weeks, 10% O_2) was also studied (20). HIF-1α protein was found in cerebral cortex of rats during the first 14 days of hypoxia falling down to normoxic levels by day 21 of the experiment. A more severe hypoxic challenge (8% O_2) after these 21 days led to re-accumulation of HIF-1α. HIF-1α protein was detected in neurons, astrocytes, ependymal cells and possibly in endothelial cells and the mRNA up-regulation of the target genes glucose transporter-1 and VEGF could also be observed during this period. The authors speculate that HIF-1 triggered adaptation is able to restore normal tissue oxygen levels during hypoxia adaptation.

A well established adaptation mechanism to high altitude hypoxia is the increase of glucose utilization during both rest and exercise accompanied by a shift towards glucose metabolism (7). Considering the number of genes involved in the glycolytic pathway which are up-regulated by HIF-1, one could speculate that HIF(s) are of importance for this metabolic adaptation to acute and chronic hypoxia. However, direct experimental evidence of this connection in subjects exposed to hypobaric hypoxia is sparse due to the difficulty in obtaining human samples such as tissue biopsies.

As already mentioned, HIF-1α expression kinetics after systemic hypoxia vary according to several factors such as time, tissue studied and severity of hypoxia. In human studies, another level of complexity is added focusing on training and endurance effects of hypoxia. Interestingly, one of the few studies analyzing the induction of HIF-1α in muscle after training under hypoxic conditions (corresponding to an altitude of 3850 m) showed increased HIF-1α mRNA levels irrespective of training intensity (65, 151). This is remarkable since it is known that HIF-1α is mainly regulated –at least under physiological conditions - at the protein level (158). High intensity training in hypoxia increased mRNAs

coding for myoglobin and VEGF (accompanied by concomitant increase in capillary density) and mRNA of the HIF-1 target gene phosphofructokinase could be detected after high intensity training in both hypoxia and normoxia. Most of studies concerning high altitude hypoxia concentrate on HIF-1 target genes rather then in HIF-1α expression itself. The two most widely studied HIF-1 target genes are erythropoietin and VEGF.

Increased erythropoiesis as a consequence of enhanced erythropoietin plasma levels is the best known adaptative mechanism to high altitude hypoxia. Epo plasma levels increase in the first 24-48h of hypoxia exposure (in moderate – 1500-3000 m - as well as extreme altitudes >3000 m) up to 2.5 fold and drop back to sea levels within the following three weeks even if the subjects remain at high altitude (107). An increase of 28% in the serum erythropoietin levels was observed in human subjects after just 2 hours of exposure to hypobaric hypoxia (10%O_2 in nitrogen) (87). Remarkably, fine controlled regulation of erythropoietin expression is still observed after up to 22 years of exposure to intermittent hypoxia (between sea level and 3550 m) occurring on a weekly basis (57). The fact that regulation of erythropoietin after chronic and intermittent hypoxia is so distinct suggests a complex modulation of the expression of the erythropoietin gene in humans.

Recent animal studies have also demonstrated the complexity of erythropoietin hypoxic regulation by the HIFs particularly in the kidney. Kidney HIF-1α expression after systemic hypoxic decreased after 2 hours exposure to 6% O_2 (141). A further study (122) mapped the expression of HIF-1α (8% O_2, 5 hours) to renal tubular cells but found that HIF-2α was most prominently expressed in the peritubular interstitium. This finding led to the suggestion that HIF-2α might play a role in the regulation of erythropoietin production since erythropoietin producing cells have been shown to be peritubular localized (37, 89, 92). HIF-3α expression has also been demonstrated in kidney and could participate as a negative regulator of HIF-mediated hypoxic response and therefore in the up-regulation of erythropoietin by HIF-1 (56). Thus although HIF-1 regulation of erythropoietin under hypoxic conditions is well established at the cellular level but there is still much to be defined about how the HIFs regulate and modulate the production of erythropoietin *in vivo*.

Two of the most important pathologies related to high altitude exposure are acute mountain sickness (AMS) and high-altitude cerebral edema (HACE, which is considered a more severe form of AMS). Typical symptoms of AMS are headache, dizziness, vomiting, fatigue and insomnia (120) that possibly result from mild cerebral edema (50). Recent studies suggest that vasogenic brain edema, the translocation of proteins and fluid from the vascular space across the blood-brain barrier, is involved in the pathogenesis of HACE (50). The HIF-1 target gene VEGF has been recently shown to be involved in the pathogenesis of these two diseases (134, 163, 129). One of the most prominent characteristics of VEGF is its effect on vascular permeability. Severinghaus and Xu (134, 163) were the first ones to put forward the possible connection between brain vascular leakage and edema formation in high altitude with the production of VEGF. In agreement with this, VEGF was also shown to be expressed in the central nervous system by astrocytes (104) and in neurons after hypoxic exposure (113). Furthermore, Schoch *et al.* (129) demonstrated that inhibition of VEGF is able to prevent hypoxia-induced vascular leakage in the brain. The connection between VEGF and AMS/HACE seems to be brain specific since the correlation between plasma levels of VEGF and high altitude illness is controversial (102, 152). The participation of VEGF in another important high-altitude illness, namely high-altitude pulmonary edema (HAPE) is also not well defined and has recently been challenged (55).

The effects of chronic systemic hypoxia in the lung were studied using HIF-1α heterozygote (HIF-1α$^{+/-}$) mice. These mice develop normally and cannot be distinguished from the wild-type under acute hypoxic or normoxic conditions. Differences are found solely in adaptation to chronic hypoxia. Yu *et al.* (164) showed that after exposure to 10% O_2 for six weeks, heterozygote mice had a delayed development of polycythemia. In addition, HIF-1α$^{+/-}$ mice have decreased right ventricular hypertrophy, pulmonary hypertension and pulmonary vascular remodeling in addition to increased weight loss when compared to the wild type. This indicates that HIF-1α partial deficiency has significant effects on multiple systemic responses to chronic hypoxia. Impaired development of pulmonary hypertension was due to decreased muscularization of the pulmonary arterioles and deficient pulmonary remodeling. Shimoda *et al.* (136) further investigated the cause for the reduced pulmonary hypertension and suggested that HIF-1α plays a role in mediating both vasoconstriction and vascular remodeling observed during the pathogenesis of hypoxic pulmonary hypertension. HIF-1α$^{+/-}$ mice were also used to study carotid body function and the ventilatory responses under chronic hypoxia (88). They showed that HIF-1α$^{+/-}$ mice displayed a lack of ventilatory adaptation to chronic hypoxia due to impairment of carotid body function.

Although the critical importance of HIF-1α in the cellular (molecular) response to hypoxia and for the cellular oxygen sensing mechanism is well defined, there is still much learn about how the cellular response mediates adaptation of the whole organism. The coordination between different HIF-1α protein levels, markedly different kinetics of expression in different organs in addition to different HIF-homologue proteins only add to the overall complexity of the hypoxic response. In addition, much is still unknown about the regulation and consequences of HIF-1-induced target genes such as VEGF in physiological as well as pathological circumstances. In summary, there is still much to be discovered about the connection between cellular mechanisms of oxygen sensing, the hypoxic response and the consequences for the adaptation of the whole organism to low oxygen conditions.

Table 2. Changes in barometric pressure, oxygen partial pressure (pO_2) in dry and water-vapor saturated air (37°C) compared to height. The percentage of oxygen ($\%O_2$) required for a gas mixture to simulate the corresponding height, as well as the temperature at this height (based on a decrease of 1°C/150 m), is also given. Table kindly provided by H. Mairbäurl.

Meters	Barometric Pressure (mmHg)	pO_2 (dry; mmHg)	pO_2 saturated; mmHg)	$\%O_2$ in a mixture	°C
0	760	159	149	20.9	15.0
500	716	150	140	19.7	11.7
1000	674	141	132	18.6	8.3
1500	634	133	123	17.6	5.0
2000	596	125	115	16.4	1.7
2500	560	117	108	15.4	-1.7
3000	526	110	100	14.5	-5.0
3500	493	103	94	13.6	-8.3
4000	462	97	87	12.8	-11.7
4500	433	91	81	12.0	-15.0
5000	405	85	75	11.2	-18.3
6000	354	74	64	9.7	-25.0
7000	308	65	55	8.5	-31.7
8000	267	56	46	7.4	-38.3
9000	231	48	39	6.4	-45.0

CONCLUSION

The discovery of the mechanisms underlying responses to hypoxia in physiological as well as pathological conditions is quite recent. HIF-1α was first discovered in 1995, meaning that in less than ten years a whole new field of intensive investigation with many important therapeutic consequences has emerged. Knowledge about the HIF-pathway has provided insights into many different fields, including embryonic development, tumorigenesis, ischemic diseases and high altitude acclimatization.

HIF-1 was discovered because of its role in acclimatization to high altitude that is mediated by its target gene erythropoietin. It is known that wide physiological, cellular and systemic responses to hypoxia depend on HIF(s). Without a doubt, as research progresses, more will be revealed on the role of the HIFs in (patho)physiological conditions related to high altitude. For instance, recently the permeability effects of VEGF have been suggested to play a role in some high altitude illnesses. Even the well defined up-regulation of erythropoietin by HIF-1 may gain new aspects since a contribution of HIF-2α and HIF-3α have recently been suggested in this process (122) (56). Thus the role of HIF(s) in coordination and mediation of hypoxic responses at the cellular and at the whole organism level as well as its complex protein and signaling pathway will be continually hotly pursued. Overall, increased knowledge of the hypoxic response will open new therapeutical perspectives for the treatment of different human diseases such as stroke, wound healing and cancer.

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Chapter 8

HYPOXIA AND LUNG BRANCHING MORPHOGENESIS

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Abstract: Morphogens, growth factors and extracellular matrix (ECM) components modulate early lung branching, and have been studied extensively both *in vivo* and *in vitro*. *In vitro* studies have been particularly useful, because tissue can be manipulated either chemically or mechanically. For the most part, such studies have been conducted at ambient oxygen tensions, despite the fact that the fetus develops in a low oxygen environment. Since oxygen tension regulates the expression of various growth factors, adhesion molecules and their receptors, we investigated whether the low oxygen environment of the fetus contributes towards lung branching morphogenesis by affecting one or more these mediators. Using an established fetal lung explant model, we demonstrated that in comparison to tissues cultured at ambient oxygen concentration (21% O_2), fetal lung explants cultured at 3% O_2 show increases in terminal branching and cellular proliferation, and they display appropriate proximal to distal differentiation. To investigate the factor(s) mediating the induction of lung branching morphogenesis and differentiation by fetal oxygen tension, we focused on matrix metalloproteinases (MMPs), a group of zinc-dependent enzymes that modify ECM structure and function. Our results reveal that hypoxia suppresses MMP activity, leading to the accumulation of specific ECM components, including tenascin-C (TN-C), that act to stimulate lung branching. These studies demonstrate that low oxygen in the setting of the developing lung positively regulates lung branching morphogenesis, and suggest that the pathologic responses to low oxygen in the adult lung reflect a dysregulation of this lung developmental program.

Key Words: fetal oxygen tension, surfactant protein C (SP-C), vascular endothelial growth factor (VEGF), tenascin-C (TN-C) and matrix metalloproteinase (MMP)

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INTRODUCTION

The term 'Hypoxia' traditionally carries with it a negative connotation. Without question, hypoxia has profound physiologic effects both acutely and chronically; however, the oxygen concentration that defines 'normoxia' is tissue- and organ-specific. Although the oxygen concentration of the air that we breathe is approximately 21% (150 mmHg at sea level), the actual oxygen concentration in various cells and tissues within the body is much lower. Under normal physiological conditions, oxygen concentration in the body varies broadly from a high of 16% (100 mmHg) in the lung alveolus to below 1% (10 mmHg) within a sub-set of cells in the thymus (14). The arterial oxygen concentration of the fetus lies at the low end of the gradient, ranging from 3% to 5%. Recently investigations have highlighted the role that this low oxygen environment plays in normal fetal growth and development. Studies by our group and others suggest that low oxygen tension plays an important role in various aspects of organogenesis including cardiovascular development, lung branching morphogenesis, and metanephric vascularization.

HYPOXIA AND DEVELOPMENT

More than 30 years ago, Mitchell and Yochim reported that the embryo develops in an environment that is markedly hypoxic (28). Prior to establishing a placental source of oxygen, the embryo depends upon oxygen absorption via diffusion, and it is therefore reasonable to assume that the embryo at this stage is hypoxic. Once the utero-placental circulation is established, however, the oxygen status of the fetus is less clear. The fact that the fetus develops in an environment that is remarkably hypoxic has been all but ignored, in part, because of the very high oxygen carrying capacity of fetal hemoglobin. Tissue hypoxia can be measured using oxygen-sensing microelectrodes, but this is an invasive procedure and it is highly sensitive to sampling area and averaging techniques, making it an unwieldy tool for proper sampling of fetal oxygen microenvironments. As a more direct method of assessing hypoxic regions at the cellular level, immunohistochemical markers for hypoxia have been developed. This approach is based on antibodies raised against protein adducts of reduced 2-nitroimidazoles. Using the hypoxia marker pimonidazole, Lee *et al.* demonstrated that regions of marked hypoxia exist within the normal developing fetus *in utero* (21). The fact that the pimonidazole is effective at detecting regions of marked hypoxia (1% O_2) suggests that a range of oxygen concentrations exists within the fetus. It is important to note that this hypoxic environment exists despite the high oxygen carrying capacity of fetal hemoglobin.

Recent studies have focused on determining the role that low oxygen environment plays in specific developmental processes, especially those concerning vascularization and organogenesis (15, 22, 24, 42, 47). Investigators studying embryo development *in vitro* have established that physiologic hypoxia (3%-5% O_2) is required for normal embryogenesis (24). Chen et al observed that the low oxygen environment of the embryo acts as a physiologic signal that directs apoptosis and tissue remodeling, fundamental processes that are essential for proper morphologic development (4). Other studies demonstrate that the low fetal oxygen environment is important during organogenesis, particularly cardiovascular

and kidney. Our recent studies now show that fetal oxygen plays a role in lung morphogenesis (9).

OXYGEN CONCENTRATION AND *IN VITRO* DEVELOPMENTAL STUDIES

As stated above, the majority of cell and organ culture studies are conducted at ambient oxygen tension, which is hyperoxic for most adult tissues and for all fetal tissues (3, 4, 6, 8, 24, 29, 31, 43). *In vitro* studies of kidney, cardiovascular, and nervous system development demonstrate that the hypoxic microenvironment is a critical component of organogenesis, specifically with respect to vascularization (1, 22, 30, 37, 42, 47). Low oxygen stimulates endothelial cell proliferation and vasculogenesis, as well as epithelial cell proliferation and tubulogenesis in metanephric kidneys in culture. The effect of low oxygen on surfactant protein expression has been studied *in vitro* in late gestation fetal lungs (1), yet the role of oxygen tension in early lung branching events has not been investigated in detail.

Lung Development

Lung branching morphogenesis is initiated when a defined region of the foregut endoderm is induced to invade the surrounding mesenchyme. Thereafter, the pulmonary epithelium follows a programmed series of branching events to form the primary conducting airways and ultimately the functional gas exchange element of the lung, the alveoli (32, 45). Paralleling induction of lung epithelial morphogenesis, pulmonary vascular development is induced within the lung mesenchyme. In fact, normal airway and blood vessel development in the lung are dependent on interactions between the epithelium and mesenchyme (5, 10, 27, 39, 41, 42). Many of the growth and differentiation peptides and their receptors controlling the process of lung morphogenesis have been identified (13, 25, 32). In addition to tissue interactions and growth factors that mediate lung development, it appears that the low oxygen environment of the fetus may also be important (1). Studies by Krasnow and colleagues demonstrate that fetal oxygen tension stimulates branching of the *Drosophila melanogaster* tracheal system *in vitro* (15). Based on this information, we hypothesized that the low oxygen environment of the fetus plays an important role in mammalian lung morphogenesis.

To investigate this idea, we used an established fetal lung explant model and determined the effect of low oxygen on lung branching morphogenesis. Our studies demonstrate that culturing fetal day 15 rat lungs at fetal oxygen tension maintains lung morphogenesis (9). Lung explants cultured at 3% oxygen maintain appropriate epithelial morphogenesis as indicated by appropriate proliferation, branching, and differentiation of the lung epithelium. Briefly, fetal oxygen tension induced an increase in 3H-thymidine incorporation and increased epithelial branching in lung explants. As well, proximal distal markers of epithelial differentiation were also maintained in the 3% oxygen explant cultures. Specifically, expression of the distal epithelial marker, surfactant protein-C (SP-C) was increased two fold in 3% oxygen explants compared to ambient oxygen cultures. SP-C *in situ* hybridization demonstrates that SP-C expression is associated with distal branching epithelium. Hypoxia in adult tissues induces the expression of a wide variety of growth factors and

their receptors including vascular endothelial growth factor (VEGF). We found that VEGF expression was increased in fetal lung explants cultured at 3% oxygen and its expression was distributed throughout the developing lung epithelium and mesenchyme. These observations indicate that the low oxygen environment of the fetus plays an important role in lung branching and differentiation.

Extracellular Matrix and Development

Normal tissue morphogenesis is not only dependent upon soluble factors, but also upon each cell's ability to interact with and react to the surrounding tissue microenvironment. A key component of this microenvironment is the extracellular matrix (ECM), a complex organized network comprised of glycoproteins, proteoglycans, glycosaminoglycans and other molecules (16, 17). Tissue-specific ECM networks interact with multiple cell surface receptors to specify particular cellular morphologies and to modulate different patterns of gene expression. Accordingly, cell-ECM interactions must be precisely controlled during developmental processes to achieve proper tissue form and function. At a mechanistic level, various components of the ECM, mostly interacting with cell surfaces via integrin receptors, act to modulate proliferation and apoptosis, dictate cell shape and fate, and even cross-modulate growth factor receptors (16, 17). Since changes in cell adhesion have been linked to branching morphogenesis mediated by tissue interactions and growth factors in developing tissues and cancer (16, 17, 30, 34, 46, 48-50), we investigated the role of the ECM in our explant model of lung morphogenesis. In particular, we focussed on an ECM component that is involved in epithelial and vascular morphogenesis, namely tenascin-C (TN-C) (16, 17).

Tenascin-C

TN-C is a large multimeric molecule that assembles as a six-armed structure called a hexabrachion. Each TN-C monomer contains a region of contiguous epidermal growth factor-like (EGFL) repeats, a series of fibronectin type III domains, and a distal globular fibrinogen-homology domain. Functionally, the different domains confer TN-C with adhesive, counter-adhesive and cell signaling capabilities. Thus TN-C can activate diverse intracellular pathways, gene expression events and cellular functions. During development, TN-C is expressed at various stages of embryonic and fetal life, appearing early in a series of rostral-caudal waves that mirror temporal growth gradients that pass through the embryo. Later, TN-C is expressed in the developing skeletal and cardiovascular systems, and in branching tissues (e.g. kidney and lung), particularly at the epithelial-mesenchymal interface (46, 49). During lung morphogenesis, TN-C is expressed in a precise spatial pattern (see Figure 1), where its function has been linked to branching, since blockade of TN-C with antibodies prevents this process in isolated fetal lung explants (46). TN-C regulation and function during this process, however, has not been determined.

Of particular interest to our investigation are TN-C's ability to coss-modulate the activity of epidermal growth factor receptors (EGF-Rs) (19, 26). At a functional level, TN-C can elicit EGF-R dependent mitogenesis directly via the EGF-like repeats or indirectly through TN-C /alphavbeta3 mediated EGFR clustering. EGFR plays an important role in lung development

and it is likely that TN-C/EGFR interactions mediate certain aspects of this process (18, 40).

It is interesting to note that originally the TN-C knockout mice were thought to have no fetal, neonatal or adult abnormalities (35), however recent *in vivo* and *in vitro* systems demonstrate that this is not the case. For example, TN-C null animals not only exhibit impaired wound healing and neurological defects (23), but fetal lungs from TN-C knockout mice branch poorly in organ culture (36). Thorough investigation of each stage of lung development would likely uncover subtle, nonlethal changes in airways and vascular development. In addition, ablation of TN-C expression in remodeling adult pulmonary arteries leads to regression of vascular lesions, albeit temporarily (17). Collectively, these and other studies strongly indicate that TN-C is an important molecule to investigate in the developing lung. Our studies show that TN-C is increased in lung explants cultured at fetal oxygen concentration and that the spatial distribution of TN-C deposition is preserved by the fetal oxygen concentration. We are currently investigating the hypothesis that increased TN-C deposition mediates branching morphogenesis induced by fetal oxygen tension (see illustration Figure 1).

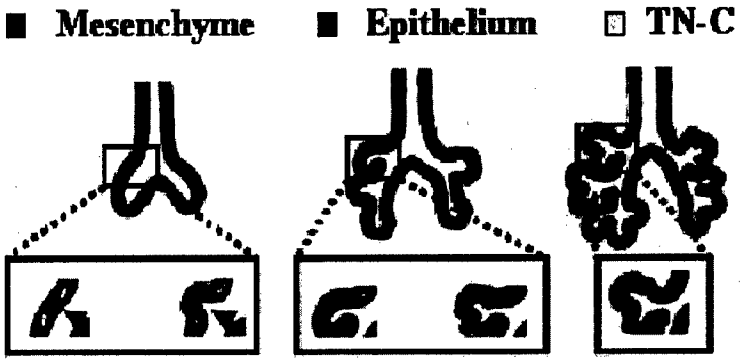

Figure 1. Role of extracellular matrix protein TN-C in primary lung branching morphogenesis. We hypothesize that foci of TN-C accumulate in spatially discrete areas restricting epithelial outgrowth in these regions. Epithelium that is not associated with TN-C deposition continues to branch into the surrounding mesenchyme. This process is repeated giving rise to an increasingly complex pattern of branched airways

Matrix Metalloproteinases

ECM remodeling is also a critical component of normal development. Local changes in the ECM alter cell migration, proliferation, and morphology and thereby regulate growth and differentiation in developing tissues (44). Matrix metalloproteinases (MMPs) are one class of proteinases that catabolize and edit various components of the ECM, including TN-C. *In vivo*, the pro-peptide is activated by proteolytic cleavage by members of the MMP family or by other proteases. MMP activity is further modulated by a family of proteins known as the tissue inhibitors of metalloproteinases (TIMPs) (2, 7, 20, 44). There are now >20 members of the MMP family; however, in the developing lung, only a limited set

of MMPs appear to be expressed, including MMP-1, MMP-2 and MMP-9 (7).

Since MMPs are important for normal branching morphogenesis and angiogenesis/ vasculogenesis, ongoing processes in the developing lung (33), we determined whether MMP activity is modulated by oxygen tension. Our preliminary studies indicate that the low oxygen environment inhibits MMP activity. Moreover, pharmacologic inhibition of MMP activity under normoxic conditions leads to increased deposition of TN-C and enhanced branching morphogenesis. Collectively, these studies suggest that TN-C protein may be accumulating at branch-points through local inhibition of MMP activity in response to hypoxia(11, 12, 38) (see illustration Figure 2).

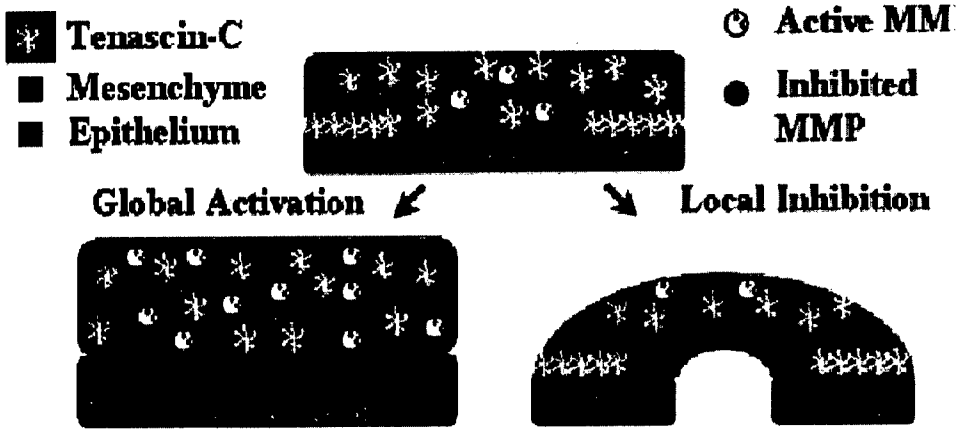

Figure 2. MMP inhibition mediates TN-C deposition. Local MMP inhibition contributes to TN-C accumulation at discrete branch points in fetal lungs cultured at 3% oxygen. Whereas, culture at 21% oxygen promotes global MMP activity, increased TN-C degradation and inhibition of fetal lung branching morphogenesis.

CONCLUSIONS

In summary, these studies provide a mechanistic framework to explain how normal low oxygen environment of the fetus promotes lung branching morphogenesis. The increase in epithelial branching is accompanied by increases in the growth factor VEGF and the extracellular matrix protein TN-C. We are currently investigating the role of hypoxia inducible transcription factor HIF-1 alpha in the hypoxic induction of VEGF in this model. Further we are investigating the role of MMP inhibition in TN-C accumulation (Figure 3). Collectively, these studies suggest oxygen concentrations normally considered 'physiologic' in the adult setting amount to hyperoxic exposure in the fetal setting. This is of particular importance in the case of preterm birth, since critical developmental processes may be impaired by exposure to the higher oxygen concentrations of either ambient air or supplemental oxygen therapy.

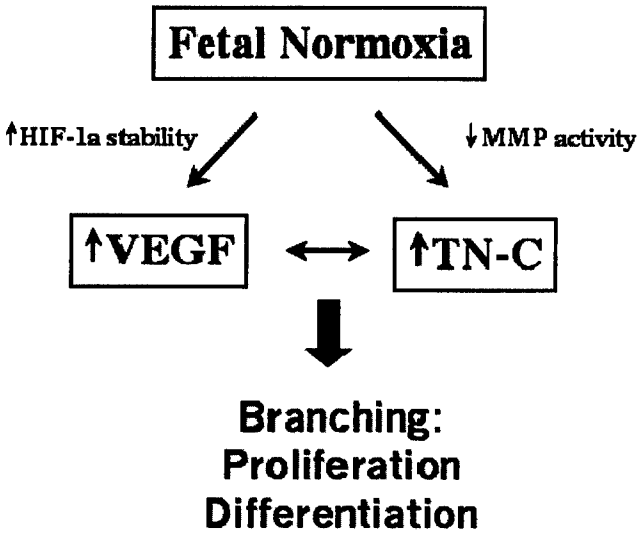

Figure 3. The normal low oxygen environment of the fetus, termed fetal normoxia, plays a central role in maintaining expression of growth factors such as VEGF and deposition of key extracellular matrix proteins such as TN-C. Collectively these factors act together to promote lung branching, cell proliferation and differentiation.

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Chapter 9

HYPOXIA AND RHO/RHO-KINASE SIGNALING

Lung development versus hypoxic pulmonary hypertension

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Abstract: Intracellular signaling via the small GTP-binding protein RhoA and its downstream effector Rho-kinase plays a role in regulating diverse cellular functions, including cell contraction, migration, gene expression, proliferation, and differentiation. Rho/Rho-kinase signaling has an obligatory role in embryonic cardiac development, and low-level chemical activation of Rho promotes branching morphogenesis in fetal lung explants. Gebb has found that hypoxia markedly augments branching morphogenesis in fetal rat lung explants, and our preliminary results suggest this is associated with activation of RhoA. Whereas hypoxia-induced activation of Rho/Rho-kinase may promote fetal lung development, other evidence indicates it has adverse effects in the lungs of neonates and adults. When exposed at birth to the mild hypoxia of Denver's altitude (5,280 ft), the neonatal fawn-hooded rat (FHR) develops severe pulmonary hypertension (PH) associated with impaired lung alveolarization and vascularization. We have observed that administration via the drinking water of the Rho-kinase inhibitor fasudil to the nursing, Denver FHR mother for the first 2 to 3 weeks, and then directly to the Denver FHR pups for the next 7 to 8 weeks, ameliorates the lung dysplasia and PH. The adult Sprague-Dawley rat develops PH when exposed for 3 to 4 wk to a simulated altitude of 17,000 ft. We have found that this hypoxic PH is associated with activation of pulmonary artery Rho/Rho-kinase and is almost completely reversed by acute intravenous administration of the Rho-kinase inhibitor Y-27632. In addition, chronic *in vivo* treatment with Y-27632 reduces development of the hypoxic PH. In summary, hypoxic activation of Rho/Rho-kinase signaling may be important for fetal lung morphogenesis, but continued activation of this pathway in the neonate impairs postnatal lung development and re-activation in the adult contributes to development of PH.

Key Words: RhoA, fasudil, Y-27632, pulmonary vasoconstriction, lung dysplasia

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INTRODUCTION

RhoA GTPase (RhoA) is a member of the Rho (Ras homologous) family of small GTP-binding proteins that includes Rac1, Cdc42, and several others. These proteins act as signal transducers that link a variety of extracellular stimuli to intracellular signaling pathways which regulate diverse cell responses, including stress fiber formation, cell contraction, adhesion, migration, gene expression, growth, and differentiation (1, 2, 8, 10). RhoA cycles between an inactive GDP-bound form and active GTP-bound form that is translocated from cytoplasm to cell membranes where it binds to and activates its downstream target proteins (effectors), such as Rho-kinase, protein kinase N, phospholipase D, diaphanous-related proteins (mDia1/2), and others. In response to extracellular stimuli such as vasoconstrictors, growth factors, cytokines, and matrix proteins, GDP-RhoA is activated by guanine nucleotide-exchange factors (GEFs) that stimulate the exchange of GTP for GDP. Other cellular signals can moderate or prevent this activation by means of guanine-disassociation inhibitors (GDIs), which inhibit the exchange of GTP for GDP, and GTPase-activating proteins (GAPs), which stimulate the hydrolysis of GTP to GDP and inactivate the membrane-associated GTP-RhoA. Greater details of the biochemistry of regulation of RhoA activity can be found in several reviews (1, 2, 8, 10, 29).

Recent reports indicate that intracellular signaling via activation of RhoA and its downstream effector Rho-kinase, i.e., Rho/Rho-kinase signaling, plays important roles in embryonic organogenesis. For example, the morphogenesis of fetal mouse hearts (54, 58) and the coronary smooth muscle cell differentiation and coronary artery formation in embryonic quail hearts (25) are dependent on Rho/Rho-kinase signaling. Similarly, Moore *et al.* report that low-level activation of Rho by cytotoxic necrotizing factor 1 (CNF-1) enhances branching morphogenesis in fetal mouse lung explants (30). In contrast to these apparent beneficial effects of Rho/Rho-kinase signaling in embryonic development of heart and lungs, there is now considerable evidence that stimulation of this signal transduction pathway in the adult has adverse cardiovascular and pulmonary effects. For instance, Rho/Rho-kinase signaling has been implicated in cardiac hypertrophy and systemic vasospasm, hypertension, and arteriosclerosis (10, 46, 51, 55), and we have preliminary evidence that it is involved in the pathogenesis of hypoxic pulmonary hypertension (PH) (9, 33, 37). Collectively, these observations have prompted us to formulate the working hypothesis that while hypoxia-induced activation of Rho/Rho-kinase signaling promotes branching morphogenesis and development in the fetal rat lung, it causes abnormal alveolarization and vascularization and PH in neonatal rats and mediates sustained pulmonary vasoconstriction and PH in adult rats (Figure 1).

HYPOXIA AND RHO/RHO-KINASE IN FETAL LUNG

As discussed in Chapter 7 of this book, Gebb has found that the branching morphogenesis of fetal rat lung explants is markedly enhanced when the explants are incubated under conditions of 3% O_2 instead of 21% O_2. Because Rho/Rho-kinase signaling is important in embryonic organogenesis (25, 30, 54, 58), and because there is evidence that hypoxia by unknown mechanisms leads to activation of Rho/Rho-kinase (40, 49, 53), we have begun to investigate the idea that this signal transduction pathway mediates the hypoxia-induced

augmentation of lung branching morphogenesis. Our only experiment to date indicates that membrane-associated RhoA, an indirect measure of RhoA activation (12), is increased in fetal day-15 Sprague-Dawley rat lung explants incubated for 48 h in 3% O_2 vs. those incubated in 21% O_2 (Figure 2). This preliminary observation agrees with the results of Moore *et al.* that low-level activation of Rho by CNF-1 enhances lung branching morphogenesis (30). Much more work needs to be done to define how hypoxia activates RhoA, and whether and how Rho-kinase, or some other RhoA effector, promotes the branching morphogenesis, but it appears hypoxia-induced activation of Rho/Rho-kinase signaling may have positive effects on fetal lung development.

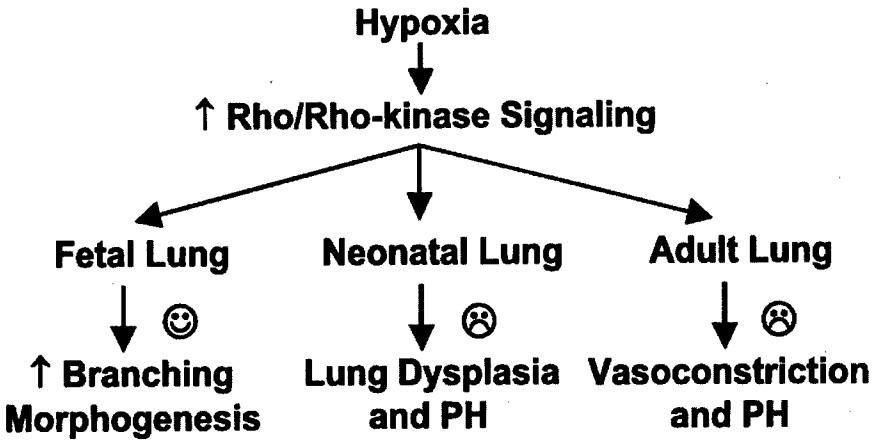

Figure 1. Working hypothesis that while hypoxia-induced activation of Rho/Rho-kinase signaling has beneficial effects in fetal rat lung by promoting branching morphogenesis, continued activation of this signal transduction pathway in neonatal rat lung causes lung dysplasia and pulmonary hypertension, and re-activation in adult rat lung mediates sustained pulmonary vasoconstriction and contributes to development of pulmonary hypertension.

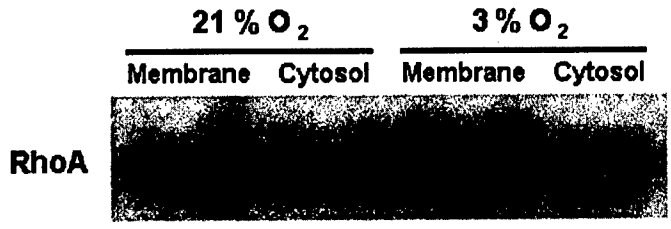

Figure 2. Western blot of RhoA in particulate (membrane) and cytosolic fractions of homogenates of fetal day-15 rat lung explants incubated for 48 hours in either 3 or 21% O_2. Levels of RhoA are higher in both fractions of hypoxic explant, and especially so in membrane fraction. This suggests that hypoxia leads to activation of RhoA.

HYPOXIA AND RHO/RHO-KINASE IN NEONATAL LUNG

In contrast to Sprague-Dawley, Wistar, Fisher, and Tester-Moriyama strains of rats, the fawn-hooded rat (FHR) has a genetic propensity to develop severe PH in the mild hypoxia of Denver's altitude of 5,280 ft (barometric pressure ~630, inspired O_2 tension ~ 120 mmHg) but not in the normoxia of sea level (inspired O_2 tension ~150 mmHg) (22, 23, 34, 43, 48). Tyler *et al.* noted that the PH in Denver FHR was associated with an emphysema-like lung morphology that included large distal airspaces ("alveolar simplification") and an apparent decrease in lung microvascular density (50). Subsequent studies have shown that the enlarged distal airspaces are not due to emphysema, i.e., not caused by an age-related destruction of alveolar walls (31), but instead are apparently due to an impairment of postnatal lung alveolarization and vascularization (22, 23).

As is the case for many mammals, the rat is born with an immature lung that must form a large number of alveoli and pulmonary capillaries to become an efficient gas-exchange organ (3, 27, 28). In the rat, the repeated subdivision of respiratory saccules and alveoli, a process referred to as septation, leads to a > 20-fold increase in the alveolar and pulmonary capillary surface areas that occurs mainly between 3 and 14 days after birth. Because FHR do not develop severe PH if exposed to mild hypoxia after 4 weeks of age (34), it is apparent the development of persistent PH in younger FHR is in some way associated with a hypoxia-induced arrest of the postnatal lung development. Hypoxia-induced lung dysplasia also occurs in neonatal Sprague-Dawley rats, but exposure to more severe hypoxia is required (26, 52). Thus, what appears to be unique to the FHR is an increased sensitivity to hypoxia. It is unknown if the adverse effects of hypoxia on neonatal lung development are elicited indirectly via nutritional or hormonal abnormalities derived from the nursing mother, or to more direct biochemical and/or hemodynamic signals in the neonatal lung.

We have so far performed two experiments to test if Rho/Rho-kinase signaling is involved in the abnormal postnatal lung development and PH of FHR born and raised in Denver's mild hypoxia. First, we compared the level of RhoA activation, i.e., membrane-associated RhoA, in lungs of 2-week-old Denver and sea level FHR. Western blotting of particulate and cytosolic fractions of Denver and sea level neonatal FHR lung homogenates indicated that RhoA activity was higher in the Denver group (Figure 3). Second, we examined effects of 10 weeks of treatment of Denver FHR with the Rho-kinase inhibitor fasudil (also referred to as HA-1077) (42, 46). Fasudil was administered via the drinking water (20 mg/100 ml) to the nursing mother for the first 2 to 3 weeks after birth and then, as the rat pups began to drink the water, directly to the pups for the next 7 to 8 weeks. As reflected in lung histology, mean pulmonary artery pressure (PAP), and right ventricular hypertrophy, treatment with Rho-kinase inhibitor ameliorated the lung dysplasia and PH in the Denver neonatal FHR. Figure 4 shows representative histological sections of lungs from 10-week-old control and fasudil-treated Denver FHR. Lungs of fasudil-treated FHR had evident increases in number of alveoli and small pulmonary arteries as compared to untreated rats. Correspondingly, the severity of pulmonary hypertension was markedly reduced in fasudil-treated FHR (PAP was 32 ± 2 mmHg in fasudil treated, n = 5, vs. 54 ± 9 mmHg in controls, n = 4, $P < 0.05$, and the ratio of right ventricular weight over left ventricular plus septal weight, RV/LV+S, was 0.39 ± 0.01 in fasudil rats vs. 0.57 ± 0.04 in controls, $P < 0.05$).

Additional experiments are required to test whether or not a higher dose of fasudil will completely prevent the lung dysplasia and PH in mildly hypoxic neonatal FHR. Similarly,

much more work is necessary to elucidate the mechanism(s) by which inhibition of Rho-kinase "protects" neonatal lungs from the adverse effects of hypoxia. At this point, it is possible that inhibition of Rho/Rho-kinase signaling alters the nutritional or hormonal status of the nursing mother's milk, or directly impacts neonatal lung epithelial, endothelial, and/or vascular smooth muscle cell differentiation, apoptosis, proliferation, and/or migration and, therefore, septation. Another possibility is that because Rho/Rho-kinase signaling in vascular smooth muscle causes sustained vasoconstriction (see below), persistent hypoxia-induced activation of Rho/Rho-kinase in neonatal FHR pulmonary arteries inhibits the pulmonary vasodilation and marked increase in blood flow that are required for conversion of the high resistance-low flow fetal pulmonary circulation to the low resistance-high flow neonatal pulmonary circulation and the normal process of postnatal lung development (6, 13, 14).

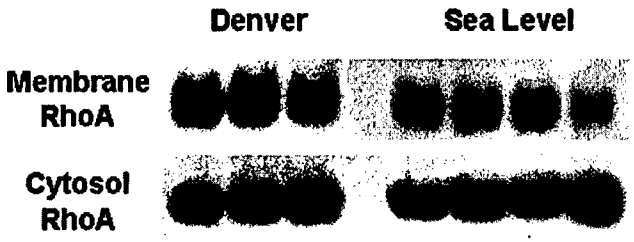

Figure 3. Western blots of RhoA in particulate (membrane) and cytosolic fractions of lungs from 2-week-old Denver (n = 3) and sea level (n = 4) FHR. Membrane-associated RhoA appears higher in Denver FHR suggesting the activation of RhoA.

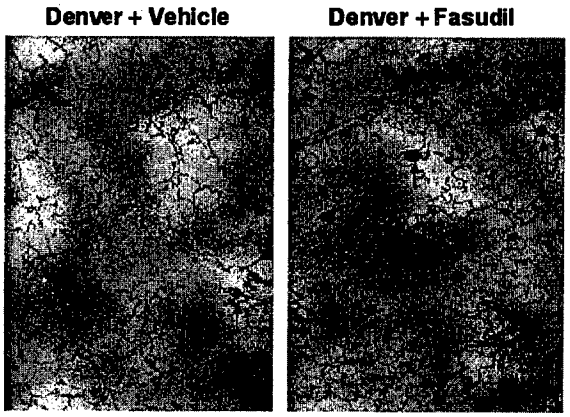

Figure 4. Photomicrographs of H&E-stained histological sections of lungs from 10-week-old vehicle- and fasudil-treated Denver FHR. Lungs were infused via the pulmonary artery with barium-gelatin before fixation. Lungs from Rho-kinase inhibitor-treated FHR show markedly improved alveolarization and increased pulmonary artery density (arrows mark barium-gelatin filled arteries) (magnification = 40x).

HYPOXIA AND RHO/RHO-KINASE IN ADULT LUNG

Hypoxic PH contributes to the morbidity and mortality of adults with various lung and heart diseases (17, 19, 57). The pathogenesis of hypoxic PH comprises an early, sustained vasoconstriction and progressive structural remodeling of the pulmonary arteries that is characterized by medial and adventitial hypertrophy of muscular arteries, and development of medial smooth muscle in the normally nonmuscular small arteries and arterioles. A current concept is that both the sustained vasoconstriction and arterial remodeling involve increased activity of various vasoconstrictors/co-mitogens and decreased activity of various vasodilators/anti-mitogens (4, 19, 57). The vasoconstrictors implicated in hypoxic PH include endothelin-1 (ET-1), serotonin (5-HT), angiotensin II (A-II), and thromboxane A2 (TXA2). The vasodilators considered to be at least relatively deficient include nitric oxide (NO) and prostacyclin (PGI_2).

Vascular smooth muscle cell contraction is generally dependent on the level of regulatory myosin light chain (MLC) phosphorylation that, in turn, is regulated by the activities of MLC kinase (phosphorylation and contraction) and MLC phosphatase (dephosphorylation and relaxation). It has recently become appreciated that smooth muscle cell contraction and vasoconstriction depend not only on an increase in cytosolic Ca^{2+} and Ca^{2+}/calmodulin-induced stimulation of MLC kinase, but also on a phenomenon referred to as Ca^{2+} sensitization (10, 38, 47). In fact, Ca^{2+} sensitization can account for sustained or progressively increasing vasoconstriction in face of a coincident fall in the level of cytosolic Ca^{2+}. There are several biochemical mechanisms of Ca^{2+} sensitization, but a major mechanism is Rho/Rho-kinase-induced inhibition of MLC phosphatase. There is evidence that G protein-coupled receptor agonists such as TXA2, ET-1, 5-HT, A-II, and norepinephrine lead to activation of Rho/Rho-kinase signaling (11, 41), and that Rho-kinase phosphorylates the myosin binding subunit of MLC phosphatase (MYPT1) and/or the inhibitory protein CPI-17, which then inhibit the phosphatase and the dephosphorylation of MLC (20, 21, 35). It is also apparent that Rho/Rho-kinase-mediated Ca^{2+} sensitization plays a critical role in the sustained phase of acute hypoxic pulmonary vasoconstriction (HPV) (40, 53). A current concept is that while an increase in cytosolic Ca^{2+} and stimulation of MLC kinase initiates HPV, sustained vasoconstriction depends on Ca^{2+} sensitization via activation of Rho/Rho-kinase and inhibition of MLC phosphatase. In contrast, it is likely that inhibition of HPV by NO and PGI_2 involves Ca^{2+} desensitization, because the downstream mediators of both vasodilators, i.e., cGMP and cAMP, can inhibit Rho/Rho-kinase signaling which leads to activation of MLC phosphatase and dephosphorylation of MLC (7, 24, 44).

Although Rho/Rho-kinase signaling plays a role in acute agonist- and hypoxia-induced pulmonary vasoconstriction (5, 18, 40, 53), and is important in the pathogenesis of various systemic vascular diseases (10, 46, 51, 55), its contribution to the pathogenesis of chronic hypoxia-induced PH is unclear. Thus, we have begun to investigate the role of this signal transduction pathway in hypoxic PH (9, 33, 37).

To test if Rho-kinase-mediated Ca^{2+} sensitization of vasoconstriction contributes to hypoxic PH, we have examined acute effects of the Rho-kinase inhibitor Y-27632 (42, 46) on pulmonary hemodynamics in adult Sprague-Dawley rats either kept at Denver's altitude of 5,280 ft (control pulmonary normotensive rats) or exposed for 3 to 4 weeks in a hypobaric chamber to a simulated altitude of 17,000 ft (chronically hypoxic pulmonary hypertensive rats) (37). We have also determined if chronic treatment of rats with the Rho-kinase inhibi-

tor attenuates development of hypoxic PH. In the first experiment, acute effects of intravenous Y-27632 (10 mg/kg) were compared in control and chronically hypoxic rats that had been returned to normoxia for 2 days for catheterization and hemodynamic measurements. Although the "chronically hypoxic" rats were no longer undergoing HPV, they maintained high pulmonary artery pressures, i.e., "residual PH", after being returned to normoxia. The Rho-kinase inhibitor had little effect on PAP and total pulmonary resistance (TPR, PAP/cardiac output) in control rats (before vs. after Y-27632: PAP = 21.4 ± 0.4 vs. 18.8 ± 0.7 mmHg, and TPR = 285 ± 6 vs. 273 ± 17 mmHg/l/min, n = 5) but markedly reversed the residual PH in chronically hypoxic rats (PAP = 35.6 ± 2.1 vs. 23.2 ± 0.7 mmHg, $P < 0.05$, and TPR = 452 ± 45 vs. 325 ± 46 mmHg/l/min, $P < 0.05$, n = 5). Y-27632 also reduced the pulmonary pressor response to an acute hypoxic challenge (10 min of 10% O_2) from 10 ± 2 to 2 ± 0.4 mmHg in control rats and from 8 ± 1 to 1 ± 0.5 mmHg in chronically hypoxic rats.

In contrast to the ability of Y-27632 to both inhibit acute HPV and reverse residual PH in chronically hypoxic rats re-exposed to normoxia, we have previously observed that the L-type Ca^{2+} channel blocker nifedipine inhibits HPV but does not reduce the residual PH (36). Collectively, these results suggest that while voltage-gated Ca^{2+} influx is necessary for ongoing hypoxic vasoconstriction (32), it does not contribute to the residual PH that exists for some time after chronically hypoxic rats are returned to normoxia. Although the residual PH has been attributed to the combined effects of hypoxia-induced vascular remodeling and polycythemia, which also regress slowly after restoration of normoxia (15, 39), the ability of Y-27632 to nearly normalize the increased PAP and TPR indicate it is due largely to Rho-kinase-mediated sustained pulmonary vasoconstriction in 3 to 4-week hypoxic rats (Figure 5). Whether or not Rho-kinase inhibition will be as effective in acutely reducing residual PH in cases of more severe and long-standing PH remains to be determined.

To evaluate if Rho/Rho-kinase signaling contributes to the development of hypoxic PH, we treated adult rats exposed to 2 weeks of chronic hypoxia with either vehicle or Y-27632 (40 mg/kg/day) via subcutaneous osmotic mini-pump. Measurements of PAP and right ventricular hypertrophy (RV/LV+S) showed that treatment with the Rho-kinase inhibitor attenuated ($P < 0.05$) the severity of PH (PAP in normoxic controls, hypoxic + vehicle, and hypoxic + Y-27632 rats = 20.7 ± 0.9, 42.0 ± 4.0, and 28.5 ± 2.2 mmHg, respectively, the corresponding RV/LV+S = 0.33 ± 0.01, 0.59 ± 0.01, and 0.45 ± 0.02, n = 3-5/group). Treatment with Y-27632 did not reduce systemic arterial pressure or alter cardiac output (not shown). It also had no effect on the hypoxia-induced polycythemia (hematocrit = 47 ± 1% in normoxic controls and 67 ± 2 and 70 ± 2%, respectively, in vehicle and Y-27632 hypoxic groups). The inhibition of hypoxic PH was only partial, and it remains to be determined if higher doses of Y-27632, or fasudil, a Rho-kinase inhibitor with possible clinical utility (46), will be more effective. These results suggest that hypoxia-induced activation of lung and/or pulmonary artery Rho/Rho-kinase signaling promotes development of PH in adult rats, and additional experiments are required to define the exact role(s) of this signaling pathway in the pathogenesis of the hypertension. In addition to mediating sustained pulmonary vasoconstriction, it is also possible that increased Rho/Rho-kinase promotes the upregulation of lung tissue ET-1 (16) and limits the expression and activity of endothelial NO synthase (49). It may also play a more direct role in the vascular cell growth (45, 56) that contributes to the pulmonary artery wall thickening.

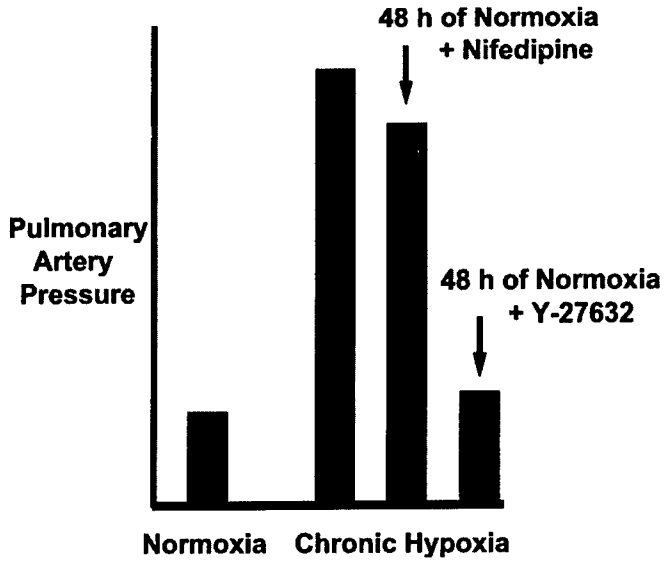

Figure 5. Demonstration of different effects of voltage-gated Ca^{2+} channel blocker nifedipine and Rho-kinase inhibitor Y-27632 on residual pulmonary hypertension in 3 to 4-week chronically hypoxic adult Sprague-Dawley rats after 48 hours of re-exposure to normoxia. While the Ca^{2+} channel blocker does not reduce residual PH (37), the Rho-kinase inhibitor almost completely normalizes pulmonary artery pressure (see text).

SUMMARY

The results of our studies suggest that hypoxic augmentation of branching morphogenesis in fetal rat lung explants is associated with activation of RhoA, that hypoxia-induced lung dysplasia and PH in neonatal FHR is associated with activation of RhoA and ameliorated by *in vivo* treatment with the Rho-kinase inhibitor fasudil, and that hypoxic PH in adult Sprague-Dawley rats involves Rho-kinase-mediated sustained pulmonary vasoconstriction and is blunted by chronic treatment with the Rho-kinase inhibitor Y-27632. Although much more work remains to be done to elucidate the mechanisms, these observations support our working hypothesis that while hypoxic activation of Rho/Rho-kinase signaling is important for fetal lung morphogenesis, continued activation of this pathway in the neonate impairs postnatal lung development and sustains PH, and re-activation in the adult contributes to development of PH (Figure 1).

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Chapter 10

HYPOXIC INDUCTION OF MYOCARDIAL VASCULARIZATION DURING DEVELOPMENT

Robert J. Tomanek, Donald D. Lund and Xinping Yue

Abstract: The development of the heart is closely linked to its temporally and spatially regulated vascularization. Hypoxia has been shown to stimulate myocardial capillary growth and improve myocardial perfusion during reperfusion in postnatal animals exposed to chronic or intermittent exposure to hypobaria. Vascular endothelial growth factor (VEGF) is up-regulated by hypoxia via HIF-1α, and these two molecules are colocalized with presumptive regions of hypoxia. VEGF up-regulation in embryonic and fetal hearts correlates with vascular tube formation which progresses from an epicardial to endocardial direction prior to the establishment of a functional coronary circulation. Our studies on explanted embryonic quail hearts indicate that vascular tube formation is enhanced by hypoxia (5-10% O_2) and inhibited by hyperoxia. Three splice variants of VEGF (122, 126, 190) were found to increase and decrease with hypoxia and hyperoxia, respectively. While VEGF synthesis is stimulated by hypoxia, there are differences in the vascular patterning between exogenous VEGF-induced vascularization and that induced by hypoxia. Thus, other, yet to be identified, molecules are recruited by hypoxia. Acute hypoxia selectively enhances at least three splice variants of VEGF-A, and also selectively up-regulates VEGFR-1 (flt-1). However, we suggest that VEGF-B, a ligand for VEGFR-1 may contribute to embryonic myocardial vascularization, since we have shown that it plays a key role in this process under normoxic conditions. A second mechanism by which hypoxia may play a role in vascularization of the heart is via its vasodilatory effects, once the coronary circulation is functional. Increased blood flow serves as a mechanical (stretch) trigger for activation of VEGF and its receptors. In sum, there is evidence that a relative hypoxia provides both metabolic and mechanical stimuli for vascular growth in the developing heart.

Key Words: vascular endothelial growth factor (VEGF), vasculogenesis, angiogenesis, hypoxia inducible factor 1 (HIF-1), VEGFR-1, quail

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INTRODUCTION

Local oxygen tension is a key regulator of the vasculature as evidenced by its role, not only in vasoreactivity, but also as a determinant of vascular growth and regression. Thus, the role of O_2 has been postulated to be a key factor in support of a metabolic hypothesis regarding tissue vascularity (1). Exposure of chick embryos to 12% O_2 during the last seven days *in ovo* caused a two- and three-fold increase in maximal blood flow to the whole body and hindlimb tissues, respectively (2). Hypoxia is known to enhance genes that encode proteins for certain cytokines, growth factors, and glycolytic enzymes. Transcription-regulating proteins are induced by hypoxia and bind to DNA sequences that control gene expression for erythropoietin, glycolytic enzymes, vascular endothelial growth factor, as well as a number of other proteins (7). Hypoxia induces the hypoxia-inducible factor 1 (HIF-1), which binds to the enhancer sequence of DNA within 5 minutes of the onset of hypoxia, attains a maximum DNA binding at 4 hr, and is eliminated 15 min after the termination of hypoxia (43).

HIF-1α is a requirement for the hypoxic induction of solid tumor formation and embryonic vascularization (34). Vascular growth in response to hypoxia is a key compensatory adaptation. This response is necessary for optimal coronary flow and reserve, and oxygenation of the myocardium during pathological states, e.g. cardiomyopathy, cardiac hypertrophy, and ischemic heart disease. Accordingly, hypoxia serves as a trigger for events that provide structural adaptations necessary for maintaining adequate tissue oxygenation. This brief review examines the evidence that the heart adapts to hypoxia by angiogenesis and that focal hypoxia during development stimulates the molecular mechanisms necessary for temporally and spatially controlled neovascularization.

HYPOBARIA FACILITATES MYOCARDIAL ANGIOGENESIS

Numerous studies have shown that chronic exposure to high altitude is associated with increases in heart mass and myocardial angiogenesis. Animals born at high altitude are characterized by a more extensive myocardial capillary growth (3, 11, 29, 30, 32, 41). An angiogenic response in the heart of animals placed in a hypobaric chamber at later postnatal time points has also been documented (14, 28, 29, 30, 41, 42). These studies indicate that capillary growth during exposure to hypobaria either fully compensates or exceeds the increase in heart mass. Although the magnitude of heart weight increases reported by various studies are variable, the major increase in heart weight occurs in the right ventricle. For example, as illustrated in Figure 1, in rats born at 5 km, right ventricular weight is three-fold higher at four weeks compared to controls maintained at 50 m (30). In the rats born and maintained at simulated high altitude, the increased heart mass was due to both myocardiocyte hyperplasia and hypertrophy and was accompanied by an increase in capillary/fiber ratio. The latter is evidence of significant neoformation of capillaries, which fully compensated for the large increase in ventricular mass. More recently Moravec and colleagues (29) reported that rats born and raised at 3.5 km for a 3 month period had right ventricular weights that were 2.6 fold greater than controls. These rats demonstrated a 34% increase in capillary density and a 31% higher value for the number of myocytes/mm^2. Thus, they concluded that cardiomyocyte proliferation accounted for the increase in RV

mass and was accompanied by capillary growth that exceeded the increase in muscle mass. Cobalt-induced polycythemia, which mimics hypoxia-induced changes, also stimulates capillary angiogenesis in the left ventricle and thereby reduces mean capillary domain, i.e. the tissue area associated with a capillary (31).

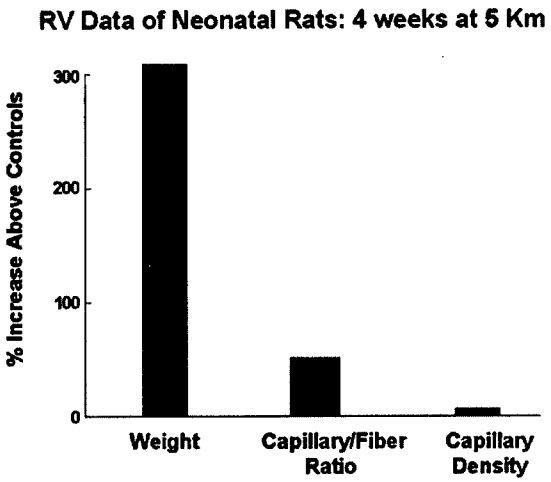

Figure 1. Effects of hypobaria on rats born and maintained for 4 weeks at 5 km. Data are expressed as percent increase over rats born and maintained at 50 m. All parameters are significantly increased compared to controls. The values are based on data from Pietsmann and Bartels (30).

Favorable adaptations in hearts hypertrophied by pressure overload, either in the spontaneously hypertensive or aortic constricted rat, occurred when the 10 week old rats were exposed to 6 weeks of simulated altitude of 4,900 m (21). In these two hypoxic groups angiogenesis was documented by an increase in left ventricular capillary density without any further increase in ventricular mass. This microvascular growth was accompanied by a decrease in heart rate and systolic arterial pressure. These findings indicate that the deficit in capillary density that occurs with pressure overload is reversed by chronic exposure to hypoxia and that the angiogenic response is not dependent on a simultaneous growth of the myocardium. This study demonstrated that the cardiomyocyte hypertrophied by pressure-overload adapts to hypoxia by increasing 1) its cell membrane area via invaginations and the number of caveolae, and 2) the number of mitochondrial profiles. Functional adaptation in hypoxia-adapted hearts is most likely related to the structural adaptations. For example, rats adapted to high altitude have been shown to recover better from episodes of acute myocardial ischemia (49) or hypoxia (35). Moreover, when a coronary artery is ligated in adapted rats mortality is reduced 5-6 times and myocardial infarct size is reduced by 35% (26). Taken together, these studies support the conclusion that hypobaric hypoxia during post-natal growth stimulates myocardial angiogenesis resulting in more extensive

capillary and pre-capillary vessels. These anatomical adaptations serve to limit myocardial ischemia.

The studies noted above have focused on animals born and reared at high altitude or exposed to high altitude for some period of time during postnatal life. To determine if fetal myocardial capillary growth was influenced by high altitude, pregnant ewes were exposed to an altitude of 3,820 m from day 30 to day 139 of gestation (20). An increase in myocardial angiogenesis was not found in the altitude group as capillary length density was slightly lower in the right ventricle which did experience a mild hypertrophy. However, capillary diameter was increased in the high altitude group. Thus, the fetus growing *in utero* at a high altitude adapts differently, than a postnatal animal. In the former the effects may not be direct. Importantly, this adaptation is geared to facilitate a greater tissue perfusion, as indicated by the finding that maximal myocardial blood flow in fetal sheep hypoxemic for 5-8 days was about 30% higher than their controls (33). This study suggests growth or remodeling of the coronary vasculature. In fetal calves right ventricular minimal coronary vascular resistance in calves kept at 3500 m was similar to that of the control group, despite a significant hypertrophy of that chamber (23). Taken together, these studies indicate that in the fetus exposed to high altitude structural adaptations occur that facilitate an increased, or at least normal, maximal myocardial perfusion that is geared to offset a lowered PO_2. Thus, this adaptation provides more O_2 to the tissue via perfusion, but does not improve O_2 diffusion distance.

Studies on hypobaria do not directly address another important issue, O_2 concentration within specific foci of the myocardium during development. Myocardial growth, prior to a functional coronary circulation, increases diffusion distances and is a likely stimulus for vascularization. This topic is addressed in a subsequent section.

HYPOXIA: A PRIMARY STIMULUS FOR GROWTH FACTORS

A major role for VEGF as a ligand facilitating hypoxia-stimulated angiogenesis is well documented in the literature (5). A number of studies have shown hypoxic induction of VEGF mRNA (10, 16, 27). VEGF's receptors are restricted to endothelial cells, while most cell types express the ligand. Thus, this arrangement is ideal for paracrine signaling from a variety of cells which become hypoxic. Moreover, the link between HIF-1 and VEGF is well established.

HIF-1 mediates the transcriptional respose to attenuated oxygen levels. This transcription factor consists of the constitutively expressed aryl hydrocarbon receptor nuclear translocator (HIF-1β or ARNT) and the hypoxic response factor, HIF-1α (43). It is the HIF prolyl hydroxalases that act as oxygen sensors and regulate HIF, and as a consequence, angiogenesis (25). HIF-1α plays a key role in VEGF release during hypoxia and is required for normal embryonic vascularization and development (34). Hypoxia has been shown to stimulate a three-fold increase in the transcriptional rate of VEGF and to enhance the half-life of VEGF mRNA by 2.5-8 fold (reviewed in 19). Stabilization of mRNA occurs with the activation of MAPKs resulting in increased expression of VEGF (4). Low O_2 tension serves to induce phosphorylation of HIF-1α by p42/p44 MAPKs. Recent evidence indicates that inhibition of HIF-1 in response to hypoxia significantly suppresses the induction of VEGF (18). Moreover, VEGF mRNA could not be induced via hypoxia in mutant cells that did not

express HIF-β (ARNT), thus further implicating HIF-1 in VEGF activation (8).

Activation of angiogenesis in response to hypoxia is organ specific, with heart and lung showing a strong response. In addition to the role played by HIF-1α, noted above, HIF-2α, a structurally related isoform, has recently been shown to play a role in cellular adaptation to hypoxia (44). Both cardiomyocytes and myocardial endothelial cells respond to hypoxia with up-regulation of HIF-2α as well as HIF-1α.

That adenosine plays a role in hypoxic stimulation of VEGF mRNA is suggested by experiments that show dose-dependent increases in VEGF mRNA in response to adenosine A2 receptor agonists, while A2 receptor antagonists reduced hypoxic stimulation of VEGF mRNA in a dose-dependent manner (36). Interleukin-1β stimulation of cardiomyoctes causes marked induction of iNOS and an even larger increase when the cells are cultured under hypoxia (13). Cardiac fibroblasts release both pro- and anti-angiogenic factors, and conditioned media from these cells added to endothelial cell cultures affects an enhancement of DNA synthesis (48). This study provided evidence that several growth factors, i.e. VEGF, PDGF, bFGF and TGF-β, contributed to the stimulatory effect on endothelial cells, and that the conditioned media facilitated the DNA synthesis in the endothelial cells during hypoxia. Hypoxia has recently been shown to enhance vascular sprout formation induced by FGF or PDGF (12). Macrophages have been shown to release PDGF and acidic and basic fibroblast growth factors when subjected to hypoxia (15). The conditioned media from these cells induced proliferation of hypoxic endothelial cells. These data support the role of a paracrine model in the hypoxic environment.

Tie-2, the receptor for angiopoietins 1 and 2, has been found to increase in coronary microvascular endothelial cells exposed to hypoxia (45). Tumor necrosis factor induced Tie-2 expression in a time- and dose-dependent manner. Interleukin-1β also increased Tie-2 expression. Such data suggest that endothelial cells respond directly to hypoxia and that inflammatory cytokines also regulate Tie-2.

While growth factors other than VEGF may contribute to hypoxia-induced angiogenesis, VEGF is clearly the key player in this response. We have suggested that vascularization of the heart is stimulated by not only metabolic influences, i.e. hypoxia, but also by mechanical stimuli, i.e. stretch (39). Both of these stimuli up-regulate VEGF, and inhibition of VEGF virtually prevents vascularization in response to either of these stimuli.

HYPOXIA STIMULATES EMBRYONIC CORONARY VASCULARIZATION

Vascularization of the embryonic heart occurs as progenitor cells from the epicardium and subepicardium differentiate into endothelial cells and form vascular tubes (vasculogenesis), which then grow by branching (angiogenesis). This tube formation occurs from an epi- to endo-cardial pattern. Our studies on rats showed that VEGF expression coincides with these vascularization processes (38). VEGF immunoreactivity was highest in the epicardium and adjacent myocardium prior to any evidence of vascular tubes. This region was the first to be vascularized. Subsequently, immunoreactivity spread toward the endocardium and was coincident with a gradient of tube formation. VEGF mRNA, visualized by *in situ* hybridization, followed this pattern. These data led us to propose that VEGF expression is dictated by a relative hypoxia, i.e. the regions farthest from the ventricular

lumen and the O_2 source express VEGF. As the compact region of the ventricle expands more regions comparatively closer to the ventricular lumen have a decreased O_2 supply and consequently express more VEGF.

The concept that increasing tissue mass causes hypoxic/nutrient-deprived cells resulting in signaling that facilitates vascularization has support from experimental data. ARNT (arylhydrocarbon-receptor nuclear translocator) is crucial in the response to both hypoxia and hypoglycemia, and embryonic stem cells in $ARNT^{-/-}$ embryos are not able to respond to low O_2 tension (22). In these embryos the angiogenic abnormalities are attributed to a failure to activate the appropriate genes, including VEGF, that facilitate vascularization. Evidence that hypoxic foci exist in the developing embryo has also been recently provided (17). Using a marker for hypoxia (pimonidazole hydrochloride), such regions were detected in the developing neural tubes, heart, and intersomatic mesenchyme in mouse embryos. Most importantly, HIF-1α and VEGF were spatially and temporally colocalized with the apparent hypoxic regions. These findings suggest that focal hypoxia plays a role in neovascularization of the developing prenatal heart.

To directly test the hypothesis that tube formation involving cardiac precursor and endothelial cells is driven by hypoxia, we utilized our quail embryonic heart explant model to investigate the effects of hypoxia on vascular tube formation (46, 47). Ventricles of 6-day-old embryos were cultured on three-dimensional gels. This culture model is characterized by migration of angioblasts and endothelial cells into the collagen matrix where they form vascular tubes. Immunofluorescence and confocal microscopy is used to visualize the endothelial cells and the vascular tubes they form by using a quail (QH1) antibody specific for endothelial cells. When cultured under 5 or 10% O_2, total tube length formed was more than twice that formed under normoxic conditions. Addition of anti-VEGF neutralizing antibodies inhibited tube formation so that tube lengths were similar to those of explants cultured under normoxia (Figure 2). Culturing the explants in 95% O_2 (hyperoxia) markedly inhibited tube formation. Thus, these data suggested a direct relationship between hypoxia and VEGF. Moreover, RT-PCR using purified RNA from heart explants showed that three major VEGF splice variants (122, 166, 190) were enhanced by hypoxia (10% O_2) and decreased by hyperoxia (95% O_2).

We then investigated the role of VEGF in hypoxia-induced vascular tube formation (47). $VEGF_{165}$ enhanced vascular growth in a dose-dependent manner; at higher doses there were fewer free endothelial cells and more tubes. In contrast, $VEGF_{121}$ had no notable effect on tube formation, a finding that indicates that its upregulation by hypoxia serves some other function in the embryonic heart. Although both $VEGF_{165}$ and hypoxia enhanced tube formation, differences in morphology were noted. Hypoxia stimulated formation of relatively narrow tubes, while addition of exogenous VEGF affected the formation of wider tubes. When the explants were exposed to both hypoxia and $VEGF_{165}$, tube morphology was intermediate to that noted with VEGF or hypoxic treatment alone. These data are summarized in Figure 3. These studies reveal that while hypoxia induces tube formation via VEGF, other factors associated with the hypoxic environment contribute to tube morphology. The differences observed could be due to the presence of other molecules induced by hypoxia, or to the effects of other VEGF splice variants or family members. Another explanation may be that hypoxia upregulates VEGFR-1 (Flt-1) but not VEGFR-2 (Flk-1/FDR) while VEGF-A upregulates both receptors (9). VEGFR-1 is a receptor for VEGF-A and VEGF-B, while VEGFR-2 is activated by three ligands: VEGF-A, VEGF-C, VEGF-D.

Hypoxia increases VEGF-A but not VEGF-B or VEGF-C (6).

Our work concerning the role of VEGF family members in vascular tube formation in quail explanted embryonic heart has documented a role of at least three VEGFs, i.e. A, B, and C (37). The finding that VEGF-B and its receptor VEGFR-1 (flt-1) play a key role in tube formation suggests that hypoxia may facilitate the role of more than one VEGF family member. As illustrated in Figure 4, upregulation of VEGFR-1 enhances its interaction with both VEGF-A and VEGF-B (as well as placental growth factor). Thus, despite the hypoxia-induced increase in VEGF-A, VEGF-B continues to compete for the VEGFR-1 and thus may play a role. The fact that other growth factors (e.g. FGFs and angiopoietins) facilitate tube formation in this model (40) underscores the complexity of the signaling required for vascularization. Thus, while VEGF family members play the central role, their effectiveness is diminished when bFGF or angiopoietins are inadequate (40).

The VEGF response to a hypoxic stimulus, however, is organ specific. Increases in VEGF and VEGFR-1 mRNAs were most marked in lung, brain, and heart, compared to kidney, testis or liver (24). Thus, the heart is able to compensate for lowered PO_2 by vascular growth.

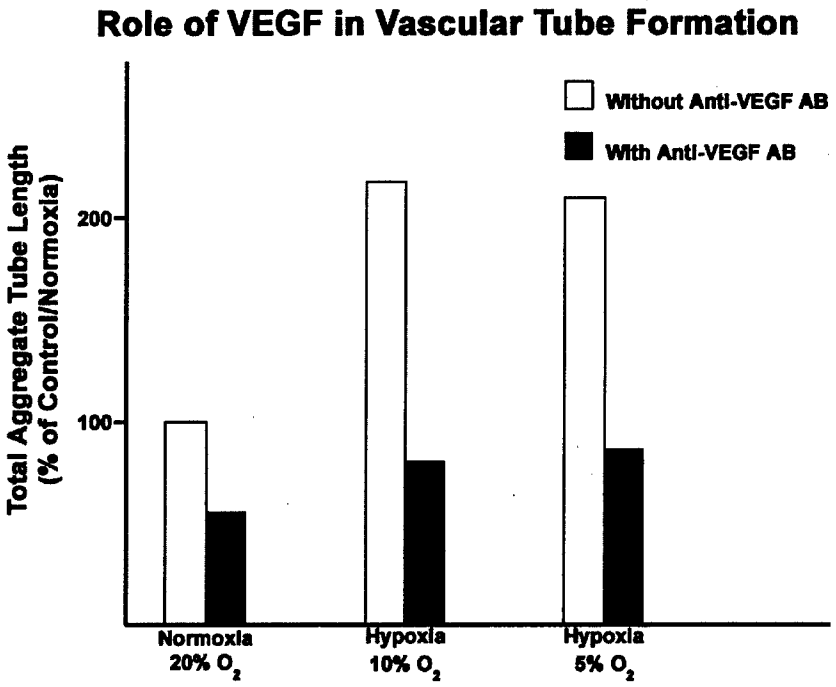

Figure 2. VEGF is required for hypoxic stimulation of vascular tube formation in explanted embryonic quail hearts. Under normoxic (20% 0_2) conditions anti VEGF neutralizing antibodies attenuate, but do not prevent, tube formation. Hypoxia, either 10 or 5% 0_2, causes a two-fold increase in tube formation, whereas anti-VEGF neutralizing antibodies completely prevent this hypoxia-associated growth. Values are based on our previously published work (46).

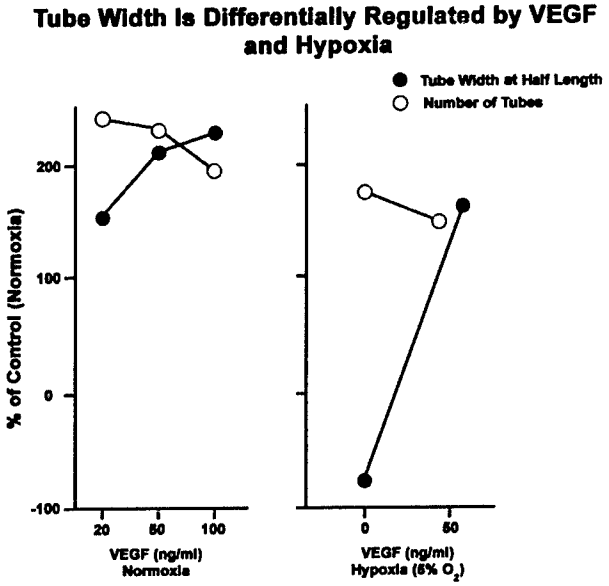

Figure 3. Tube width and the number of tubes formed on collagen gels when embryonic quail hearts are explanted. Although the number of tubes formed by addition of VEGF protein is similar to that stimulated by hypoxia, tube width is much wider with VEGF stimulation. When VEGF is added to explants cultured under hypoxia, tube width is intermediate compared to VEGF or hypoxia alone. These values were calculated from our previously published data (47).

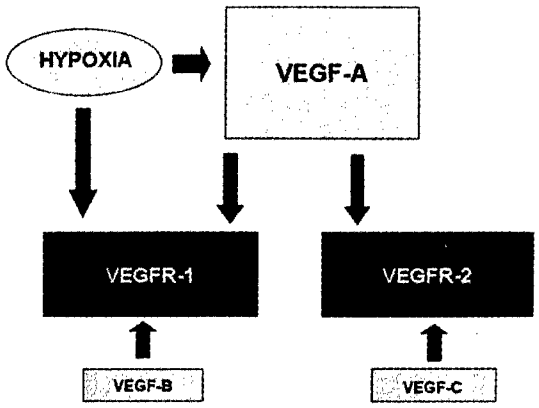

Figure 4. Relationship between hypoxia and VEGF family members and two VEGF receptors. Hypoxia has been shown to selectively increase VEGF-A mRNA and VEGFR-1 (flt-1) mRNA, but not VEGF-B, VEGF-C or VEGF-2 (Marti and Risau, 1998). This finding should not be interpreted as indicative of a single hypoxia-VEGF-A-VEGFR-1 pathway for the following reasons: 1) enhancement of VEGF-A indicates that more of this ligand is available for both VEGFR-1 and VEGFR-2 (flk-1); 2) enhancement of VEGFR-1 should allow increased binding of VEGF-B as well as VEGF-A.

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Chapter 11

ROLE OF CEREBRAL BLOOD VOLUME IN ACUTE MOUNTAIN SICKNESS

C. Mathew Kinsey and Robert Roach

Abstract: This review focuses on the role of cerebral blood volume in the intracranial hemodynamics that may influence the pathophysiology of acute mountain sickness (AMS). Cerebral blood flow is elevated in acute hypoxia exposure in humans, but the response in this setting of cerebral blood volume is unknown. After discussing the background, attention is given to noninvasive measurement of cerebral blood volume, and recent preliminary data on cerebral blood volume in AMS

Key Words: cerebral hemodynamics, blood flow, autoregulation, near infrared spectroscopy

INTRODUCTION

Since Singh's observation elevated intracranial pressure (ICP), on Indian troops ill with severe AMS(26), many authors have promoted a role for elevated ICP as the fundamental physiologic derangement leading to AMS.(12, 22). However, evidence of increased ICP in mild to moderate AMS, a condition necessary to invoke elevated ICP as a critical causal factor in AMS, remains scant. Moreover, if a rise in ICP occurs at high altitude, little is known about which mechanisms contribute to its development. Here, we review the relationship of ICP to AMS, and the role that elevated cerebral blood volume (CBV) may play in determining ICP and subsequently in the development of AMS.

ICP AND AMS

Transient or persistently elevated ICP may be the final common pathway in the development of AMS. The most consistent symptomatic feature of AMS is headache, a common finding in conditions that result in elevated ICP of any cause.(1) Several studies

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have attempted to directly address the role of ICP in the development of AMS.

In 1969, Singh and co-workers published results from studies on 1,934 Indian soldiers who were rapidly transported from sea level to 5,867 m in the Himalayas.(26) In 34 of these soldiers, lumbar punctures were performed during illness and on recovery. CSF pressures were elevated by 60 to 210 mm of water compared to recovery. Singh et al. stated that all had severe AMS, and a proportion may have actually had early HACE(26).

Hackett and Hartig measured cerebrospinal fluid (CSF) pressure, as a proxy for ICP, in three subjects decompressed in a hypobaric chamber to 5000m.(13) The decompression took approximately 3.5 hours, and the subjects were at simulated altitude for an average of 5.5 hours before they developed symptoms of AMS. CSF pressures increased slightly on ascent to 5000m, but were not correlated with symptoms. Importantly, these investigators also administered hypoxic gas under normobaric and hypobaric conditions and looked at the compensatory change in CSF pressure. Middle cerebral artery flow velocity (MCAfv) was measured by transcranial Doppler, and the change in MCAfv compared to changes in CSF pressures before and after administration of hypoxic gas (11% O2 at sea level and 16.5% at simulated altitude). For similar changes in MCAfv, these investigators found larger changes in CSF pressure upon hypoxic gas breathing at simulated altitude. They theorized that due to brain swelling, the craniospinal axis has less capacity to buffer volume changes at high altitude. In essence, the volume increase placed the system higher, shifting from A to B in Figure 1, on the non-linear pressure volume curve. It is unknown if elevated resting CSF pressure would have become apparent if the study had been continued for a longer time period, and symptoms progressed. During the development AMS, transient increases in ICP may induce symptoms, whereas later in the course of the illness or with more severe AMS (similar to the conditions under which Singh and colleagues performed measurements) elevated ICP may be persistent. If, as proposed, by Hartig et al. (13), transient increases in arterial hypertension would be transmitted to the downstream cerebral vasculature leading to concomitant short term elevations in ICP and, perhaps, the development of symptoms of AMS. An alternative explanation for the normal CSF pressured measured by Hartig and Hackett has been offered by Krasney(16) who proposed that lumbar CSF pressure may in part reflect a compensatory caudad displacement of CSF.

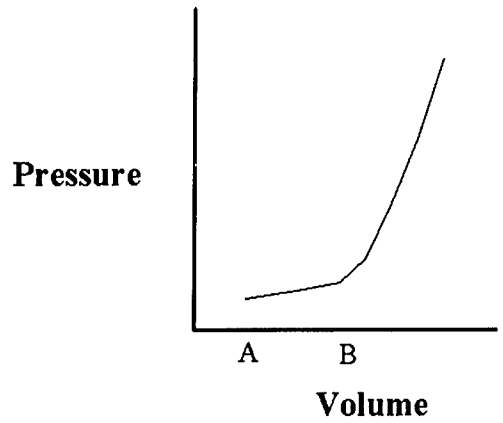

Figure 1. The pressure-volume curve for intracranial hemodynamics.

It seems probable, given the impressive results of Singh et al.(26), that AMS may be related to elevated ICP. However, transient or persistent intracranial hypertension has yet to be demonstrated in the development of AMS. Performing measurements of ICP non-invasively and under hypoxic conditions have proven technically and experimentally difficult up to this point. However, new techniques for measurement of intracranial elastance using MRI(2) may allow further understanding of the role that ICP plays in the pathogenesis of AMS.

CBV AS A PATHOGENIC MECHANISM IN AMS

Elevated CBV has been proposed as a mechanism leading to intracranial hypertension and the subsequent development of AMS. The concept of elevated CBV contributing to intracranial hypertension is not without precedent. The relative contributions to ICP by each of the volumetric spaces within the cranium (blood, CSF, brain parenchyma), in the setting of differing pathologies, is a topic of ongoing research. As the brain and its surroundings are enclosed within a rigid shell, an increase in volume of one space must be at the expense of the others. This simple relationship is referred to as the Monroe-Kellie doctrine(14, 20):

$$V_{intracran} = V_{blood} + V_{brain} + V_{CSF} + V_{mass\ lesion}$$

Several studies have implicated elevated CBV as a major component of the cerebral vasculature which is poorly regulated under pathologic conditions.

Grubb and co-workers studied CBV in 30 patients with subarachnoid hemorrhage secondary to ruptured aneurysm, but with normal ICP.(9) They used injection of the intravascular tracers $C^{15}O$, $H_2{}^{15}O$, and ^{15}O-oxyhemoglobin, and positron emission tomography to measure regional CBF and mean vascular transit time, and to calculate CBV. In patients with severe clinical symptoms (a minimum of drowsiness, confusion, and mild focal deficits) and vasospasm documented by angiography, dramatic increases in CBV were reported (approximately 45%, compared to patients without vasospasm and with the mildest symptoms). Interestingly, CBF dropped significantly compared to patients with mild symptoms and no vasospasm, demonstrating an uncoupling of CBF and CBV (this relationship will be discussed further later). Grubb et al. propose that elevated CBV in patients with vasospasm may be a response to a reduction in cerebral perfusion pressure, introducing the idea that elevated CBV may be an advantageous compensatory mechanism; with the deleterious effect of elevating ICP in patients with more severe pathology.

Applying the same techniques to primates, Grubb et al. showed that artificially induced elevations in ICP resulted in increases in CBV in monkeys.(10) They infused artificial saline to produce roughly 20 mm Hg increases in ICP. They found that CBV increased significantly over baseline at each step until an ICP of approximately 80 mm Hg was reached. At this point CBV levels remained elevated at approximately 50% above baseline, despite further increases in ICP up to 100 mm Hg. This again illustrates that regulatory mechanisms in the cerebral vasculature will effectively sacrifice further increases in ICP to maintain perfusion pressure and oxygen delivery by elevating CBV.

Further investigation of this relationship has been performed in animal models of traumatic brain injury. Twelve swine were subjected to right frontal barotrauma injury.(4) This model of injury had been previously shown to induce elevations in ICP while avoiding changes

in cardiac and respiratory rate that would occur secondary to brainstem involvement. ICP was measured using a ventricular catheter, CBV by reflected red and IR plethysmography, and brain compliance by single bolus injection of saline. They found elevations of ICP to 24 mm Hg (baseline 9 mm Hg) within minutes of injury. Interestingly, ICP returned to baseline levels within thirty minutes and slowly increased again (to levels similar to the early elevations) over the next 5 hours. CBV mirrored the kinetics of ICP change and doubled during the initial ICP rise (8.9 to 19.2 ml/100g). Although, CBV had a kinetic relationship to ICP, it was significantly elevated throughout the six-hour study period. Additionally they reported decreased brain compliance at six hours post injury, compared to pre-injury levels.

However, the role that CBV plays in intracranial hypertension following traumatic brain injury has recently been questioned by Marmarou et al.(17) In thirty one subjects with recent head injury measurements of brain edema (MRI), CBV, and ICP (ventricular catheter) were measured. CBV was established using computerized tomography by first measuring CBF using Xe inhalation and comparing it with mean transit time measured by venous injection of iodinated contrast. Results showed that brain tissue water increased on average to 79% from a baseline in normal volunteers of 77% per gram of tissue. CBV on the other hand, actually fell by an average of 0.8%. However, there was a wide variation in the post injury timing of when the imaging studies were performed, with the majority occurring 3-5 days following the initial insult. It seems plausible that CBV may undergo initial elevations following traumatic brain injury, inducing increases in ICP, but over the following hours to days extravasated fluid becomes the dominant contributor to brain swelling.

Elevated CBV has also been reported to contribute to ICP in the syndrome of benign intracranial hypertension, also known as pseudotumor cerebri(19). This is a syndrome of elevated ICP without apparent etiology. It may be precipated by transverse sinus thrombosis, or medications, but most commonly has no known causative association. It generally resolves spontaneously after several months. In two patients diagnosed with pseudotumor cerebri CBV was calculated using the injected radioactive tracers, ^{133}Xe to measure cerebral blood flow and ^{99}Tc to measure mean transit time, both during the episode and following resolution several months later. CBV was increased on average by 85% and was found to be normal following resolution of headache, resolution of papilledema, and reduction of ICP.

How could CBV influence ICP in the development of AMS? Marmarou has proposed that much of the volumetric buffering that occurs in the cranial vault may be secondary to compressibility of the more compliant venous vessels.(18) Following bolus injection of saline in normal adults, buffering of the additional fluid occurs almost instantaneously and the increase in ICP is transient. These authors propose that the only component of the neural axis capable of responding with this rapidity is the vascular compartment. Thus, the pressure-volume index (PVI) is not determined by the mass of neural parenchyma. This idea comes from a study of 34 severely head injured patients (GCS<8) in which the relative contributions of the vascular and CSF compartments to elevated ICP were calculated.(18) Dynamic measurements were made by rapid bolus addition and withdrawal of CSF, allowing calculation of the pressure volume index and assessment of CSF formation and outflow resistance. Using these parameters, the relative contributions of each of the compartments could be calculated. They found that the vascular compartment accounted

for approximately 66% of the rise in ICP, and concluded that the compliant vascular vessels serve as a buffering mechanism as they are compressed by brain volume increase.

Elevations in CBV would be expected to decrease brain elastance, as can be seen in the pressure-volume curve in Figure 1. Since 70% of the total CBV is contained in the venous vessels, volumetric increases in this vascular bed would decrease the compliance of these vessels leading to increased brain stiffness (a decreased PVI) and an effective shortening in the flat part of the pressure volume curve, the distance from A to B in figure 1. Thus, elevated CBV would alter the pressure volume relationship by increasing brain stiffness as well as increasing total volume within the cranial vault and thereby contributing to elevated ICP.

CBF AND CBV

CBF is elevated in response to acute hypoxia, but does not correlate with the development of AMS.(5) However, the role of CBF in elevation of CBV has not been explored in hypoxic humans. Several studies have addressed the role of CBF in the regulation of CBV. In 1974, a seminal study reported results from work in primates showing that dramatic changes in CBF resulted in only modest changes in CBV.(11) Grubb and co-workers simultaneously measured CBF and CBV in rhesus monkeys using radioactive ^{15}O. CBV varied approximately as the cube root of CBF with induced changes in pCO_2. This was the first study to reject the idea that CBV and CBF were linearly related. As discussed previously, Grubb also showed that in 30 patients with subarachnoid hemorrhage CBF was decreased in the setting of increased CBV.

In a more recent study, Fortune and co-workers verified this relationship in humans using technetium labeled red blood cells to measure changes in CBV in eight healthy volunteers. (8) They compared changes in CBV to concomitant changes in CBF, measured by duplex scanning of the internal carotid artery, under conditions of hypocapnia, hypercapnia, and hypoxia. Although CBV and CBF tended to change in the same direction, they did not track proportionally. This was particularly true under hypocapnic conditions, where CBF decreased by 31% but was matched by a 7% change in CBV, compared to normocapnia. Interestingly, significant hypoxia (average SaO_2 = 76.7%), under normobaric conditions, produced an 11% change in CBF and a 5% change in CBV.

HYPOXIA AND CBV

Hypoxia, even under normobaric conditions, can induce symptoms of AMS.(23) Could a 5% change in CBV (as seen by Fortune and co-workers) result in elevated ICP and subsequently cause the symptoms of AMS? Elevated CBV, as mentioned previously, may alter both brain volume and brain stiffness. Discounting any contribution of increased CBV to brain stiffness, a 5% change in CBV could be expected to add approximately 4 mls of blood volume to the brain (assume 1500g brain at 5 ml blood per 100g tissue). Because the pressure volume curve in figure 1 is exponential, it can be transformed into a linear equation by plotting it on a semilogarithmic scale. The slope of the resultant line yields an index of compliance which is independent of ICP and is generally referred to as the pressure volume index (PVI). The PVI can be viewed as the theoretical volume (in ml) to be added to the CSF space to obtain a tenfold increase in pressure; the normal value is 25 ml.(25) Given a

normal PVI of 25 ml, a 3.75 ml increase would result in a mild transient pressure increase to approximately 27.5 mm Hg. (15±12.5 ml) This small volume would be rapidly buffered by vascular compression. However, a 4 ml volume addition to the craniospinal axis would occupy a large portion of the spatial reserve capacity (approximately 6ml). Further increases in CBV or tissue edema would then lead to large and persistent increases in ICP, relegating the role of elevated CBV to that of a necessary but not sole condition for the development of AMS if craniospinal compliance is similar. Several authors have suggested that a) human craniospinal compliance varies widely, and b) that for AMS pathophysiology a large craniospinal compliance may be protective, and a small craniospinal compliance deleterious. Alternatively, the alteration of brain stiffness by CBV may be such that the relatively flat portion of the pressure volume curve is shortened, allowing small volume increases in CBV to significantly alter ICP.

ROLE OF CO_2

A hallmark of successful altitude acclimatization is a marked ventilatory response resulting in a respiratory alkalosis, largely due to hypocapnia. The importance of this stimulus is highlighted by the fact that one of the actions of acetazolamide, the drug most commonly used to prevent altitude sickness, is to increase blood CO_2 levels by inhibiting the formation of bicarbonate. How does altered pCO_2 affect CBV? Fortune et al. showed that CBV was more sensitive to increases in pCO_2 than to decreases.(8) The results of Rostrup et al. further support the idea that CBV changes are more sensitive to hypercapnia than hypocapnia.(24) These investigators measured CBF with ^{15}O labeled water and CBV with both positron emission tomography (PET) and the total hemoglobin near infrared spectroscopy (NIRS) method in five healthy subjects. Hyperventilation and inspiration of 6% CO_2 were used to induce changes in $PaCO_2$. During hypercapnia, they reported an average increase in CBF of 37% and an increase in PET CBV of 29%. However, during hypocapnia CBF decreased by an average of 25% while PET CBV was not significantly different from normocapnic conditions. As proposed by Fortune et al., these studies imply that there may exist a "tonic vasoconstriction" in the brain. How this state affects CBV response in the setting of hypobaric hypoxia is unknown.

ADDITIONAL REGULATORY MECHANISMS

The relationship between CBV and mean arterial pressure has been studied in piglets. Tsuji et al. measured regional cerebral oxygen saturation using NIRS, CBV using the NIRS total hemoglobin method, and CBF with radioactive microspheres during 3-4 minutes of hypotension to approximately 50% of baseline.(27) Even under these dramatic conditions, CBV only fell by an average of 11 μmol/L while cerebral regional oxygenation decreased by an average of 65 μmol/L. Thus, under normal conditions, CBV appears to be maintained despite marked hypotension. However, little is known regarding CBV changes under conditions of impaired cerebral autoregulation, such as has been postulated to occur at high altitude.(12, 22)

Several known vasodilators have been studied as mediators of CBV. Nitric oxide (NO) has been well established as a mediator of large cerebral artery diameter, but only a few studies have addressed its role in regulation of basal tone of small intraparenchymal

vessels. Kobari et al. used a photoelectric lamp implanted through the skull to assess cortical Hb concentrations, a proxy for CBV in cats.(15) The NO synthase inhibitor L-NMMA was infused at varying dosages while changes in CBV were monitored. CBV decreased within the first minute and continued to fall over the fifteen minute measurement period. This effect was dose dependent with a maximal fall in CBV of approximately 2% with a dose of 0.7 mg/kg/min of L-NMMA. Importantly, the observed decrease in CBV could be competitively inhibited if L-arginine, the natural substrate for NO synthase, was infused before L-NMMA. Interestingly, hypoxia has been shown to be a strong stimulus to the production of NO.(3)

Adenosine has also been investigated as a mediator of CBV. Newman et al. infused adenosine into in utero sheep fetuses and monitored CBV using NIRS and CBF with a transonic carotid monitor.(21) They found that CBV increased rapidly during adenosine and reached a maximal average increase of 18 μmol/L above baseline by the end of the twenty minute infusion period. CBV subsequently returned to baseline values within thirty minutes of stopping the infusion. Adenosine is a breakdown product of ATP and could therefore be elevated under hypoxic conditions where ATP production by the electron transport chain would be inhibited. Thus, several mechanisms seem reasonable candidates to account for CBV changes in an environment of altered CO_2 and O_2 levels, but none have been studied in acute hypoxia in intact humans.

PRELIMINARY RESULTS: CBV IN AMS

We recently measured CBV by the near infrared spectroscopy total hemoglobin method in one volunteer before and after 9 hrs at 4600 m.(6, 7, 28) The subject developed a marked headache and other symptoms of AMS. Compared to control conditions CBV was elevated during high altitude headache/AMS. During the final measurements we administered oxygen for five minutes and observed a drop in CBV and notable clinical improvement. We did not make simultaneous measurements of CBF, and thus do not know the extent of CBF changes with the onset of headache, or its resolution by oxygen. These results suggest a potential pathogenic role for elevated CBV in high altitude headache, the cardinal symptom of AMS. Further studies are needed to elucidate the role of CBF in the observed CBV changes.

SUMMARY

A case has been presented for a role for elevated cerebral blood volume in the pathogenesis of AMS. New, non-invasive techniques should allow simultaneous measurement of cerebral blood volume and blood flow in subjects ill with AMS, and during recovery. If CBV elevation is shown to track the onset of AMS then further studies will be needed to determine the pathogenic mechanisms at play.

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Chapter 12

VENTILATION, AUTONOMIC FUNCTION, SLEEP AND ERYTHROPOIETIN

Chronic mountain sickness of Andean natives

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Abstract: Polycythemia is one of the key factors involved in the chronic mountain sickness syndrome, a condition frequent in Andean natives but whose causes still remain unclear. In theory, polycythemia may be secondary to abnormalities in ventilation, occurring during day or night (e.g. due to sleep abnormalities) stimulating excessive erythropoietin (Epo) production, or else it may result from either autogenous production, or from co-factors like cobalt. To assess the importance of these points, we studied subjects with or without polycythemia, born and living in Cerro de Pasco (Peru, 4330m asl, CP) and evaluated the relationship between Epo and respiratory variables both in CP and sea level. We also assessed the relationship between sleep abnormalities and the circadian rhythm of Epo. Polycythemic subjects showed higher Epo in all conditions, lower SaO_2 and hypoxic ventilatory response, higher physiological dead space and higher CO_2, suggesting ventilatory inefficiency. Epo levels could be highly modified by the level of oxygenation, and were related to similar directional changes in SaO_2. Cobalt levels were normal in all subjects and correlated poorly with hematologic variables. The diurnal variations in Epo were grossly abnormal in polycythemic subjects, with complete loss of the circadian rhythm. These abnormalities correlated with the levels of hypoxemia during the night, but not with sleep abnormalities, which were only minor even in polycythemic subjects. The increased Epo production is mainly related to a greater ventilatory inefficiency, and not to altered sensitivity to hypoxia, cobalt or sleep abnormalities. Improving oxygenation can represent a possible therapeutic option for this syndrome.

Key Words: hypoxic ventilatory response, chemoreflex, baroreflex, autonomic, nervous system, polycythemia, sleep disturbances

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INTRODUCTION

High altitude (HA) natives show erythrocitosis which can be termed "normal" or "physiologic". This is in general moderate in well adapted subjects, and follows a linear relationship with the altitude of residence (43). Nevertheless, many HA natives become severely symptomatic from "excessive" erythrocitosis (EE), a condition called chronic mountain sickness (CMS) or Monge's disease. The problem of why EE develops only in some but not in other HA residents of the same altitude is not fully understood. Essentially, the basic mechanism is that hypoxia may not be completely counterbalanced by appropriate ventilation (in absolute or in relative terms). The resulting hypoxemia in turn stimulates the production of Epo that results in polycythemia. This apparently simple mechanism, however, may be altered at different levels, each of them may lead to EE. Several of these alterations are schematically shown in Figure1.

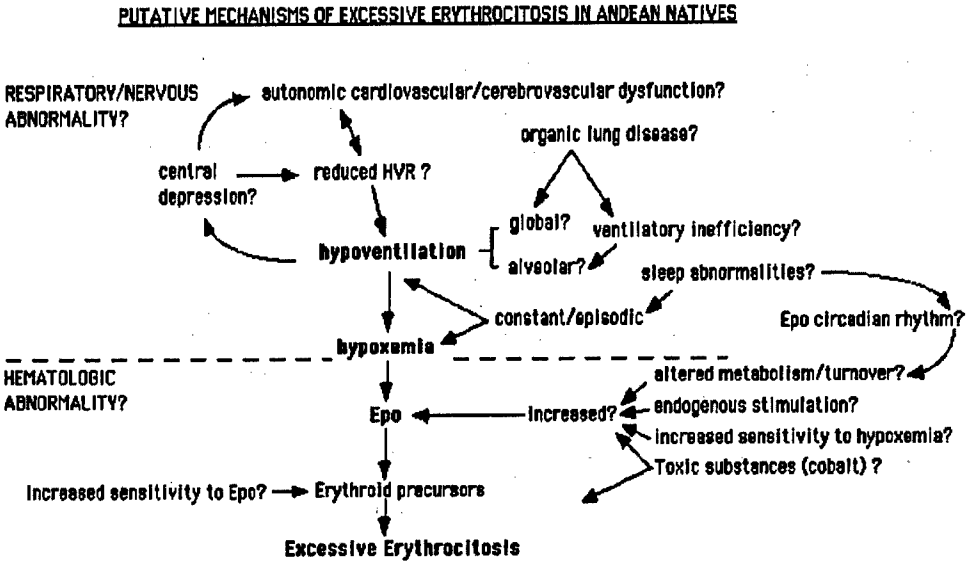

Figure 1. Putative mechanisms of excessive erythrocitosis in Andean natives.

This schema does not take into account other potentially important factors, like physical exercise and pulmonary hypertension, whose importance still needs to be examined by future studies.

The inappropriate ventilation may result from a reduced ventilatory response, leading to hypoventilation, or to inefficient ventilation, for example resulting from a high ventilation/ perfusion inequality and/or increased dead space, or else be the result of an underlying chronic respiratory disease, or even be the consequence of abnormalities occurring during sleep, and finally, be a consequence of one or more of these factors.

However, the production of Epo can be exaggerated in response to a standard hypoxic

stimulus, or else the hemathologic effects of Epo can be favoured or "amplified" by cofactors (like for example cobalt) known to stimulate erythropoiesis. The sensitivity to Epo of the erythroid process can be enhanced, and finally a feedback mechanism that can reduce the production of Epo can be non operant in these subjects. In this review some of the relationship between ventilation and Epo are examined. We will also examine recent findings that may have relevance in understanding some of the physiopathologic aspects of this disease.

ABNORMAL VENTILATION AND EXCESSIVE ERYTHROCYTOSIS

The increased Epo production and the consequent polycythemia may be secondary to abnormalities in ventilation, in turn stimulating excessive Epo production. This may include organic lung dysfunction of any type, either obstructive, restrictive or affecting diffusion capacity. The frequent occurrence of polycythemia in miners has made this hypothesis quite plausible. At high altitude, even a mild respiratory dysfunction may have much worse consequences and hence lead to chronic hypoxemia.

High altitude Andean residents with EE have lower oxygen saturation as compared to resident without EE. This finding is confirmed by most of other studies on CMS (39, 34), and it is often found to be associated with an increased PCO_2 end tidal, suggesting functional hypoventilation, or, alternatively, some degree of organic lung dysfunction. Indeed, a variable degree of lung dysfunction has been demonstrated in subjects with EE, while mild degrees of respiratory dysfunction may even remain unrecognised, but it is clear that the disease occurs even in the absence of overt lung disease. For example, Leon-Velarde *et al.* (34) found that subjects with chronic lower respiratory disease (assessed by low peak expiratory flow rate) had significantly higher frequencies of CMS score (41.2%) and EE (32.4%) as compared to normal subjects (25.0 and 11.3%, respectively), and concluded that at high altitude chronic lower respiratory disease is strongly associated with the development of EE, while acute or chronic upper respiratory disorders are not. However, it was also clear from this study that respiratory diseases are not the only cause of EE and CMS, as an excess hemoglobin was present also in a substantial proportion of normal subjects.

This again leads to the hypothesis that alveolar hypoventilation, of whatever origin, either functional or secondary to organic disease, may play a role in the origin of EE. Because ventilation is under control of reflex mechanisms, and, particularly at altitude, is stimulated by hypoxia, one may relate the hypoventilation to a blunted hypoxic ventilatory response, which is a rather common finding of native subjects of high altitude. The concept that the hypoxic ventilatory response is blunted even in well adapted HA natives (48, 49), has been recently reconsidered by newer evidences, showing that HA natives, particularly in Tibet and in the Himalayas, appear to have a normal hypoxic ventilatory response (HVR) (40). Nevertheless, all reports confirm that Andean HA natives with EE have reduced ventilatory drive, at least in comparison to matched HA natives without EE. The question, however, is whether a reduced hypoxic drive is really the cause of hypoventilation. In fact, many reports showed the coexistence of reduced hypoxic drive but comparable resting ventilation values in HA subjects with EE (19). In a preliminary study (8) we raised the question as to whether a reduced hypoxic ventilatory response is indeed evidence of hypoventilation. In

a group of Andean natives of Cerro de Pasco we examined the relationship between HVR and resting ventilation, under hypobaric hypoxa (in Cerro de Pasco), right after 45min of hypobaric normoxia (by oxygen administration in Cerro de Pasco) and under 24 hour of normobaric normoxia (in Lima, sea level). In these subjects we found that resting ventilation increased, whereas HVR decreased during exposure to normobaric normoxia or did not change after short term oxygenation. These results indicate that resting ventilation is not simply dependent on the slope of HVR, but from the entire position of the curve. In fact, while the slope dropped, the intercept remained the same, implying that the right side of the curve was shifting upward (ie to a higher level of ventilation for the same amount of oxygen saturation). Two types of considerations could be drawn from those data: 1) chronic hypoxia was depressing resting ventilation: 2) a blunted HVR does not necessarily mean diminished resting ventilation and consequent hypoxemia, but has to be regarded as a response to an acute hypoxic challenge. The consequences are that functional ventilatory abnormalities can be present and play an important role in EE.

Chronic Hypoxia Depresses Resting Ventilation

A central depression has been repeatedly reported after exposure to hypoxia (57, 48). Even after acute exposure to hypoxia minute ventilation first increases rapidly, then, after 5-10 minutes, it drops by 15-20%, though still remaining above baseline levels (57).

Subjects with CMS do have both hypoxemia and depressed ventilation. As a consequence, they have been hypothesised to have central depression. This is partly confirmed by the findings that respiratory stimulants improve long term ventilation and reduce EE (29). Short-term administration of oxygen has been shown to increase minute ventilation in HA natives (48, 27, 46, 54, 60), further suggesting that respiratory depression may be an important factor in the origin of the hypoxemia. In Andean high altitude natives of Cerro de Pasco we have found that 45 minutes of oxygen administration at altitude and 24 hour in normoxia at sea-level both increase minute ventilation, thus clearly indicating that central depression can be present in these subjects. This also implies that improved oxygenation would restore normal ventilation and reduce central depression, thus interrupting a sort of vicious circle which reinforces hypoxemia and hypoventilation despite the increase in hemoglobin.

In our subjects we found a continuum of values, indicating that central depression was not a specific feature of those subjects with higher EE, but was present also in subjects without polycythemia. This finding does not support the hypothesis that EE can be the result of a specific genetic defect affecting a subgroup of Andean population, but rather is an evidence of different degrees of maladaptiation to high altitude, and that, if a genetic predisposition does exist, it may rather affect the entire Andean population. This is confirmed by studies (59) showing that overall, Andean natives tend to have much higher levels of hemoglobin and hematocrit as compared to subjects living in the Himalayas at comparable altitudes.

Blunted HVR Does Not Necessarily Mean Diminished Resting Ventilation And Consequent Hypoxemia

Subjects with optimised respiration (as a practice of yoga) have a depressed HVR (51, 5), but a highly efficient ventilatory pattern and no hypoxemia during acute exposure to simulated altitude (6). Similarly, highly adapted HA populations may have reduced HVR and no hypoxemia, again indicating a compensation by more efficient ventilation at rest. Preliminary results from our group, obtained in subjects in Cerro de Pasco with and without EE, indicate an increase in blood oxygenation, without an increase in minute ventilation by adopting a yoga-derived type of breathing in HA subjects with EE (7, 28). The possibility that this may be a useful strategy was indirectly confirmed by observation of one subject, in whom we observed a quite peculiar behavior. This subject was breathing spontaneously at slow rate, he had a rather low minute ventilation, a quite depressed HVR, but oxygen saturation was normal, Epo levels, hemoglobin and hematocrit data were also in the lowest range of normals. This pattern was similar to that predicted by a long-term practice of yoga. When questioned, the subject reported that he had a long-term practice of Karate in Cerro de Pasco, and that he had made practice of slow breathing techniques, which turned out to be similar to those of yoga. In previous studies we reported that the main advantage of a slow and deeper breathing is an improvement in ventilation/perfusion inequality, with reduction of the physiologic dead space (indirectly evidenced by a lower Vd/Vt ratio) (4) . Our preliminary findings in Cerro de Pasco evidence that the Vd/Vt ratio is higher in subjects with EE, and these values correlate with blood hematocrit and hemoglobin concentration (7).

These findings and considerations indicate that indeed Blunted HVR does not necessarily mean diminished resting ventilation, consequent hypoxemia, and ultimately EE.

Evidence Of Functional Respiratory Abnormalities In Excessive Erythrocytosis

In subjects free from major respiratory diseases, the coexistence of normal (or near normal) resting ventilation, reduced SaO_2 and increased CO_2-et suggests reduced alveolar ventilation, increased physiological dead space, and this has been indeed shown by a number of studies on HA-EE (18, 36, 24). The relieving effect of hemodilution has been attributed to an improvement of ventilation/perfusion mismatch, as, despite a decrease in blood haematocrit and haemoglobin concentration it decreased the alveolar-arterial gradient, and increased the PaO_2, oxygen saturation and ventilation (36, 18). HA natives from Tibet, supposed to have a good adaptation to high altitude have been shown to have a lower alveolar-arterial gradient (61).

These findings indicate that subjects without evident pulmonary diseases may still have a functional pulmonary abnormality. In order to test whether this abnormality correlated with blood abnormalities we correlated and index of ventilation/perfusion abnormality (the Vd/Vt ratio) and hemathologic data, and found that subjects with higher blood levels have also an increased Vd/Vt ratio. In turn both series of data were also correlated to the levels of oxygen saturation and end-tidal carbon dioxide. These findings have practical implications: we know that certain types of ventilatory patterns improve the ventilation/perfusion

inequality, or else increase the alveolar ventilation at the expenses of a reduction in physiological dead space. For example, the complete yogic breathing improves the ventilation/perfusion inequality and increases oxygen saturation in heart failure (4), a condition often characterised by increased ventilation/perfusion inequality (4) and reduced diffusion (2). In the same group of subjects with or without EE we have examined the effects of slow breathing on these values. We found a marked increase in oxygen saturation, which was not due to an increase in minute ventilation, again suggesting an increase in alveolar ventilation and an improvement in ventilation/perfusion inequality (7).

AUTONOMIC INVOLVEMENT OF CARDIOVASCULAR AND RESPIRATORY FUNCTIONS AT ALTITUDE IN CHRONIC MOUNTAIN SICKNESS

There is no information as to the autonomic control of cardiovascular and cerebrovascular functions at altitude in CMS. The question is relevant, because it is well known that there is a strict interrelationship between the control of blood pressure and respiration. In addition, alteration in the autonomic control can affect the production of Epo, as it normally occurs in dysautonomic subjects (11). Finally, some of the clinical symptoms of CMS appear to be related more to autonomic dysfunction and, possibly, cerebrovascular dysregulation, than to the polycythemia.

In a series of research studies we have evaluated the baroreflex function and the chemo- and baro-reflex interactions in subjects with and without EE, we also analysed the relationship between hemathologic parameters and autonomic function, evaluated the cerebrovascular function in these subjects, and tested whether oxygen administration, either passive or self-administered, could relieve possible autonomic abnormalities.

Our first studies clearly identified that, together with a reduction in the peripheral chemoreflexes, Andean subjects with EE also show a reduction in the baroreflex control of heart rate and blood pressure (9). In our subjects we have found that the reduction in the arterial baroreflex correlates with the increase in CMS score and with hemoglobin levels in Cerro de Pasco. This simultaneous reduction in both chemo-and baroreflexes is at variance with the well known inverse relationship that exists between chemo- and baroreflexes in healthy subjects at sea level (50). In some pathologic conditions, like chronic congestive heart failure, there is an imbalance characterised by an increase in chemo- and a reduction in baroreflexes (40); this alteration is functional and reverses with clinical improvement. Therefore, in our Andean subjects with EE the simultaneous depression of both chemo- and baroreflexes could be interpreted as a sign of an organic dysautonomia or of a central depression. However, when our subjects descended to Lima, at sea level, we observed an evident increase in arterial baroreflex, together with an increase in minute ventilation, thus suggesting that central depression was implicated in the abnormality seen at high altitude. Furthermore, the increase in baroreflex occurred with a parallel drop in the CMS score, due to a reduction in clinical symptoms. These findings suggest that the observed alteration in the baroreflex can be implicated in the origin of some symptoms of CMS. Thus, improving oxygenation restores the arterial baroreflex and reduces symptoms of CMS, by a likely mechanism of relieving the central depression. How can oxygenation be given at

high altitude? In a subsequent study we compared the effect of 1 hour of passive oxygen administration with 1 hour of self oxygenation obtained by slow breathing (6 breaths/ minute), and assessed the effects on the arterial baroreflex. Both techniques increased oxygen saturation and increased the arterial baroreflex, indicating that self-oxygenation could be effective in relieving central depression and improving the cardiovascular and respiratory function (28). Finally, preliminary studies indicate that in the presence of hypobaric hypoxia the cerebrovascular sensititvity to carbon dioxide is impaired in subjects with CMS, thus suggesting another possible link between the autonomic disturbances and the origin of symptoms (44). In conclusion, autonomic disturbances are present in Andean altitude natives with EE and CMS. These abnormalities have influence on both the respiratory control and are also likely to be implicated in the origin of some symptoms of CMS. Improved oxygenation restores baroreflex function and the reciprocal relationship between chemo- and baroreflex, and improves clinical CMS symptoms.

ERYTHROPOIETIN IN HIGH ALTITUDE NATIVES WITH AND WITHOUT EXCESSIVE ERYTHROCYTOSIS, POSSIBLE DETERMINANTS

It is well known that serum Epo rapidly increases during hypoxia induced by HA exposure; however, with continuous exposure serum Epo falls in the normal range (37), or even to levels that are undetectable by *in vivo* assays (1). The fall in serum Epo occurs before the hemoglobin concentration has reached its new steady-state value, and therefore, at a time when hemoglobin is continuously raising. A feedback activated by the increase in hemoglobin (3, 56) and by a reduction in plasma volume (22, 45, 27, 47, 53), which occurs early in the acclimatization process to high altitude, probably accounts for a reduction in Epo, even before a real erythrocitosis is taking place. As a result of this feedback mechanism in normal or in anemic subjects at sea level, there is an inverse hyperbolic relationship between haemoglobin or haematocrit and Epo (32)

Winslow *et al.* (59), and, similarly, Schmidt *et al.* (47), have shown that a higher hematocrit is maintained, at high altitude, with a lower proportion of Epo, at least in subjects with an appropriate altitude-induced erythrocitosis. These observations suggest that, at a physiological level at least, an increased level of hemoglobin can be maintained in men chronically exposed to high altitude with only a small increase, if any, of serum Epo (33). This conclusion underlines the problem of establishing a relationship between variables with greatly different time constants: Epo has a delay of about 2 hours (21) and a half-life which has been estimated in man at 1.5-3 hours (32), whereas the different steps of erythrocitosis need days (accelerated maturation of reticulocytes) or weeks. The data available so far about the relationship between Epo and EE/CMS are limited, and there is still no evidence of a strict relationship between Epo and EE. Winslow *et al.* (59) measured the concentration of serum Epo in Andean natives and Sherpas living at 3700m, and found that Andeans had higher hemoglobin levels and also serum Epo level concentrations. Leon-Velarde *et al.* (33), studying 61 high altitude Andean (19 of whom had EE) and 20 sea-level natives, found that the concentration of Epo in HA natives was significantly higher than that in SL. However, the Authors could not find a significant difference between HA sub-

jects with or without EE, suggesting that in these subjects small increases in Epo may be enough to induce a marked erythrocitosis. The fact that Epo values were similar in spite of a large (22%) difference in hemoglobin is difficult to be explained on the basis of the current available literature.

This implies that other factors are modifying the simple relationship between Epo and hemoglobin. It is possible that Epo levels may be increased at certain times of the day or the night in reponse to particular stimuli and then return to normal levels at the time when blood samples are taken. If this is the case then the crucial point is to determine the circadian rhythm of Epo at high altitude and see whether this rhythm is different in EE as compared to normal subjects. This circadian rhythm has never been done at altitude, nor a search for its possible determinants was attempted. Sleep abnormalities, if present, are likely to induce hypoxemia and Epo stimulation. In principle, one can also hypothesise that the sensitivity to Epo can be increased in subjects with EE, thus allowing a greater hemopoietic stimulation for the same level of Epo. Finally, it is also possible that the effects of Epo could be somewhat amplified by exogenous substances known to affect hemopoietic function. Increased cobalt levels have been recently suggested to play an important role in EE (31).

Altered Circadian Rhythm Of EPO

Epo levels have a circadian rhythm, and this can be altered in subjects with EE. Previous studies have identified a circadian rhythm of Epo, with peak at early night/late afternoon, and a progressive decrease during night (38, 58, 15, 26). Therefore, the values obtained in the morning, when blood samples are normally obtained, are expected to be the lowest of the entire day, despite a change in even 60% are to be expected during daytime (58). If the daily profile of Epo is maintained also at HA and in subjects with EE, then it is possible that an increased peak cannot be seen. So far the daily profile of Epo has never been determined in HA residents, nor any study have attempted to measure if subjects with EE have an altered daily profile of Epo. We have reported the first observation of circadian Epo variations in high altitude natives. In a group of Andean subjects natives of Cerro de Pasco, and without EE, we have found a circadian rhythm of Epo almost identical to that reported at sea level (58), with a zenith during later evening and a nadir at 8:00 AM (10). The Epo values were similar to those reported at sea level, and similarly, there were ample circadian variations, reaching, in the average, 40% from day to night. This finding alone can thus explain a possible major source of variability of Epo data, as the time at which the blood sample is taken has a major impact on the Epo levels. But the most interesting finding was the fact that the circadian rhythm was almost completely disrupted in the subjects with EE, also native and resident in Cerro de Pasco. At any time, the Epo values were much higher in EE as compared to the non EE subjects, and the day-night variation was abolished. Under these conditions, it was evident that abnormalities occurring during both day and night were responsible for the alterations found. This indicated that alterations occurring during sleep could have been responsible for part of the elevations in Epo (for example during late night and early morning), but it also pointed out that, in order to maintain a sustained elevation in Epo, hypoxemia should have been present also during the day.

Sleep Abnormalities

In some subjects, Epo levels can be higher during the night and stimulate erythropoiesis, but return to normal during the day, despite EE. Night variations may be due to sleep disturbances of various types from obstructive sleep apneas to simple hypoventilation. These have been reported in very small groups of high altitude natives from China and residents in Tibet (55) and in 5 subjects from Leadville (29, 30). Instead, the few data available so far from sleep studies carried out in Andean altitude natives seemed to show a lower frequency and extent of abnormality (41, 16, 17). Furthermore, in many of these studies the method used was not standardized, so that in conclusion, although it has been suggested that sleep abnormalities are indeed crucial in order to explain the EE at high altitude (43), convincing evidence is in fact lacking. A comparison of all studies published (to our best knowledge) on high altitude natives, reveals that so far only a very limited number of subjects with chronic mountain sickness have been studied (8 subjects were studied in refs. 16 and 17, but CMS was not assessed, 5 subjects in refs. 29 and 30, 8 subjects in ref. 55, 14 subjects in ref. 41). Data available are thus based on 22 CMS subjects worldwide and results are discordant. Instead, several studies consistently found frequent sleep abnormalities in lowlanders visiting high altitude places, but these findings cannot be applied to subjects permanently living at high altitude.

To answer this question we have carried out sleep studies at high altitude, together with the circadian rhythm of Epo, in a group of subjects with and in a group of subjects without EE, all native and resident in Cerro de Pasco. Despite the high levels of Epo in the EE group, we did not find major sleep abnormalities. Simple and occasional hypopneas were the most frequently abnormalities found, however, these abnormalities were equally frequent in both EE and control subjects. The only evident difference was the presence of a consistent reduction in oxygen saturation, which reached its minimum between 2:00 and 3:00 AM. The difference in oxygen saturation between the two groups was really moderate, in the range of 3% only. However, very interestingly, the values in the EE group were consistently around or below 80% during the entire night period, whereas in the control groups most of the time was spent above 80%. We also found a significant correlation between the oxygen saturation values found during night time and the Epo levels seen during the morning, indicating that these relatively small changes could have been indeed relevant in determining the increased Epo levels of our subjects. There was no or much lower correlation between morning Epo levels and the amount of time spent at lower levels of oxygen saturation.

These findings in part seem to confirm (to the extent that data are comparable) previous observation made in Andean natives, which tended to exclude an important role of sleep abnormalities in these subjects. It is also confirmed that oxygen saturation plays an important role, but the data also suggest that it is not necessary to reach a very low level of saturation. In a previous experimental study it was found that a critical PaO_2 or SaO_2 level was necessary in order to trigger the increase in Epo levels. This value was found to be 80% for SaO_2 (and 50 mmHg for PaO_2) (14). This Figure is identical to the values found in our study (52) for the EE group, and clearly points to a threshold level below which Epo start increasing. It also explains well why these two groups were so much different in terms of Epo levels, despite only a minor absolute difference (around 3%) in oxygen saturation.

Altered Sensitivity To EPO Of The Erythrocytotic Process

EE may result from an increased sensitivity of erythroid cell precursors to Epo. Nevertheless, preliminary results from our group (13) showed that the growth of erythroid progenitors (BFU-E, burst-forming units erythroid) was similar in HA subjects with EE, in normal HA natives, and in healthy sea-level native controls, both in terms of Epo-dependent and Epo-independent proliferation. This indicates that at least some of the erythropoietic process occurs normally in subjects with EE, and excludes that an increased sensitivity to Epo can be leading to sustained stimulation of erythrocitosis.

Exogenous Substances Stimulating Erythropoiesis

Cobalt has been shown to induce erythropoiesis by a mechanism that in part shares some similarity to hypoxia (20), so the presence of toxic levels of cobalt in areas of high frequency of chronic mountain sickness may be a potentially important factor. A recent study (31) reported toxic levels of cobalt in subjects with EE; we have determined cobalt concentration in a similar group of subjects from the same city, but failed to confirm these findings (35), so the possible role of cobalt probably requires further definition but it is not likely to explain the occurrence of EE, except perhaps in selected cases.

RESPIRATORY-HEMOPOIETIC INTERACTION

The results seen so far indicate a link between the abnormalities in ventilation and hemopoiesis. A practical question is whether this information can be used to modify the consequences of respiratory abnormalities seen in CMS.

Can EPO Production Be Reduced By Improved Oxygenation In High Altitude Natives With Excessive Erythrocytosis?

This question is highly relevant in te context of EE. If Increased Epo levels result from an autogenous increased secretion it is unlikely that increased Epo levels could be normalized by an increasing blood oxygenation. Instead, if increased Epo levels are the consequences of hypoxemia, then increasing blood oxygenation should suppress Epo production. This simple idea, however, is complicated by the fact that while the time constant of the increase in Epo in response to hypoxia is known (32) there is only a very limited amout of information about the opposite aspect, i.e. the kinetics of Epo reduction (if any) in response to oxygenation.

There are very few studies that analysed the time course of Epo in chronic hypoxia after oxygen administration (23, 25), so it is not well known what should be the best time lag to observe a change, if it does occur. However, changes in Epo levels, after a period of hypoxia, are detected in the blood after 1-1.30 hours (21). The reversibility of the high levels of Epo in HA natives with EE, and its time course, is also not well known. Faura *et al.* (25) reported a drop in Epo levels in 6 HA residents of Morococha after descendng to Lima, however there was not enough information to draw conclusions about the time

course of the Epo drop.

We exposed subjects with and without EE to 45 minutes of oxygen administration. The oxygen flow was regulated in order to keep the oxygen saturation similar to the levels commonly observed at sea-level (about 96%). Epo levels were measured 2 hours after cessation of he oxygen administration. Epo levels were markedly reduced in all subjects, with or without polycythemia, at this time interval (12). Because we had only one point of observation it is not possible to establish the time course of Epo in response to oxygenation, nor whether this was simply the beginning of the effect or its maximum. We could also not be able to establish if all subjects, for example with or without polycythemia, had the same time course in response to oxygenation. These are essential aspects that need to be answered and have important practical consequences. If the reduction in Epo tends to last several hours it may be possible to administer bouts of oxygen for limited periods of time, whereas if the response is limited in time, then oxygen saturation (or PaO_2) should be maintained higher for a longer period of time. Our current research is aimed to answer these fundamental physiopathologic and practical aspects.

CONCLUSIONS

Whether it is clear that the presence of organic lung diseases, of whatever type, can induce polycythemia at high altitude, it is nevertheless evident that this can occur also in the absence of any overt respiratory disease. Available data concur to indicate that inefficient ventilation, perhaps associated to a reduced ventilatory drive, could be responsible for chronic hypoxemia and increased Epo levels. At the opposite, an increase in oxygen saturation induces a marked reduction in Epo levels. The correlation of Epo levels and other hematologic variables with oxygen saturation and with high carbon dioxide levels argues against a decisive role of other factors not linked to respiration. If increased Epo were the effect of autogenous stimulation we would not see any correlation with respiratory variables, nor increased oxygenation would likely be able to reduce Epo levels. Similar considerations apply to exogenous toxic substances. There is no support for a major role played by sleep abnormalities for the development of EE in HA Andean native subjects.

In conclusion, currently available information tend to suggest that the increased Epo production is mainly related to a greater ventilatory inefficiency, whereas pulmonary dysfunction, sleep abnormalities, and toxic factors may be implicated in selected cases, but do not appear to represent the main, general cause of the disease. This conclusion leads to practical consequences, as improving oxygenation may potentially reverse the chain of events leading to polycythemia and CMS.

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Chapter 13

CARDIO-PULMONARY INTERACTIONS AT HIGH ALTITUDE

Pulmonary hypertension as a common denominator

Marco Maggiorini

Abstract: The purpose of this review is to find the evidence that a disproportionate pulmonary vasoconstriction persisting for days, weeks and years during residence at high altitude is the common pathophysiologic mechanism of high altitude pulmonary edema (HAPE), subacute mountain sickness and chronic mountain sickness. A recent finding in early HAPE suggests that transmission of excessively elevated pulmonary artery pressure to the pulmonary capillaries leading to alveolar hemorrhage as the pathophysiologic mechanism of HAPE. The elevated incidence of HAPE in Indian soldiers led the Indian Army to extend the acclimatization period from a few days to 5 weeks. Using this protocol, HAPE was prevented, but after several weeks of residence at an altitude of 6000m dyspnea, anasarca and pleuro-pericardial effusion developed. Clinical examination revealed severe congestive right heart failure. This condition has been previously described in long-term high altitude residents of the Himalaya and the Andes. In rats, smooth muscle cells appear in normally non-muscular arterioles within days of simulated altitude. Rapid remodeling of the small precapillary arteries may prevent HAPE but increase pulmonary vascular resistance leading to pulmonary hypertension in long-term high altitude residents. Symptoms and signs of HAPE, subacute mountain sickness and chronic mountain sickness reverse completely after residents are transfered to low altitude. In conclusion, these findings strongly suggest that pulmonary hypertension at high altitude, which could be named "high altitude pulmonary hypertension", is the principal and common pathogenic factor of all three cardio-pulmonary manifestations of high altitude illness. Accordingly, subacute mountain sickness and chronic mountain sickness could be renamed in "acute-" and "chronic right heart failure of high altitude", respectively.

Key Words: high altitude, pulmonary hypertension, high altitude pulmonary edema, subacute mountain sickness, chronic mountain sickness, Monge disease, right heart failure

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INTRODUCTION

The physiologic response of the pulmonary circulation to hypobaric and normobaric hypoxia is to increase pulmonary arteriolar resistance. The magnitude of hypoxic pulmonary vasoconstriction is highly variable between humans, probably based on genetics and adaptive mechanisms. Sites of hypoxic pulmonary vasoconstriction are small pulmonary arterioles and veins of a diameter less than 900 μm, the veins accounting approximately for 20% of the total increase in pulmonary vascular resistance caused by hypoxia (4, 14). The structural changes in small pulmonary arteries and veins appear to reflect this genetically based and adaptive process (6, 10, 32) in humans and animals. Excessive hypoxic pulmonary vasoconstriction (HPV) and thus susceptibility to develop a right heart failure at high altitude has been described in Indian soldiers stationed at an altitude between 5800 and 6700 m (1) and in Han infants in Lhasa (46) as well as in the Colorado cattle. In cattle, this clinical entity has been named "Brisket disease" because edema developed in the depending part of the neck that is called "brisket" (15). Recently, selective breeding of cattle for low and high hypoxic pulmonary vasoconstrictor response has wiped out Brisket disease in the Colorado cattle (6), suggesting the genetic basis of the disease. In humans, reports showing that in the Andes, susceptibility to high altitude pulmonary edema (HAPE) runs in families (20)and that Tibetans, who are the best adapted population to high altitude, have virtually abolished HPV (10) suggest the genetic and evolutionary influence of HPV.

Excessive HPV has not only been reported to cause high altitude cor pulmonale within weeks, months, or years in newcomers and in high altitude residents of the Andes, but it is also a hallmark in not acclimatized climbers who develop high altitude pulmonary edema (HAPE) (42). Therefore, it is reasonable to assume that an excessive rise in pulmonary artery pressure (Ppa) is the common denominator of HAPE, the syndrome first described by Sui in infants and by Anand in adults and termed "subacute mountain sickness" of the infant and the adult, respectively, and in the illness of the high altitude residents of the Andes termed chronic mountain sickness or "Monge disease". We cannot exclude that all these individuals may share common, at the present time unknown, genes that influence the magnitude of their pulmonary arterioles to respond to hypoxia.

In this review we discuss different aspects of diseases associated with pulmonary hypertension at-high altitude according to their pathophysiologic mechanisms and clinical presentation in the different subjects and setting. Moreover, based on their different pathophysiological and clinical aspects we propose a new classification, in which the elevated pulmonary artery pressure is the common link. In fact the terms "subacute mountain sickness" and "chronic mountain sickness" are misleading. The name "subacute mountain sickness" was originally used by Carlos Monge to describe persistent symptoms of acute mountain sickness – for weeks and months after arrival at high altitude (29, 31), which is not associated with pulmonary hypertension. Similar confusion exists between the terms "Monge disease" and "chronic mountain sickness". The disease described for the first time by Monge in high altitude residents of the Andes is a syndrome characterized by excessive erythrocytosis, profound hypoxemia and pulmonary hypertension (28, 29). Unfortunately, the term "chronic mountain sickness" has been used to describe both, high altitude excessive erythrocythosis in South-Americans and congestive failure of the right heart in the Himalayas (9, 33, 35). Most of these high altitude residents do not present with the trias of excessive polycythemia, severe hypoxemia and mental retardation.

EFFECTS OF ACUTE EXPOSURE TO HIGH ALTITUDE

In healthy lowlanders at altitudes between 3800 and 4600 m, invasively assessed resting mean pulmonary artery pressures (Ppa) range between 15 and 35 mmHg (average 25 mmHg) and the systolic Ppa between 27 to 48 mmHg (average 37 mmHg) (24, 26, 50) (Figure 1). At altitudes above 5000 m, the mean Ppa was assessed during a right heart catheterization in 6 resting healthy acclimatized volunteers during Operation Everest II. At a barometric pressure of 347 Torr (6100 m) and at 282 Torr (7620 m) the mean Ppa was on average 19 mmHg and 34 mmHg, respectively (Groves *et al.*, 1987). At both altitudes physical exercise significantly increased Ppa being at 347 Torr on average 41 mmHg and at 282 Torr 54 mmHg (11).

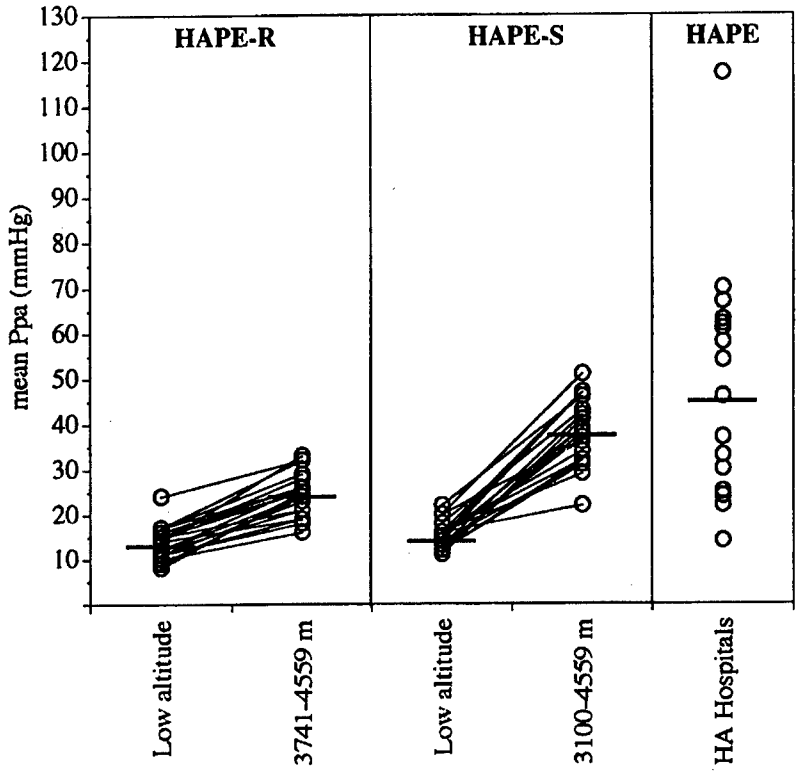

Figure 1. The Figure shows individual pulmonary artery pressure (Ppa) values reported at low and approximately 24 hours after ascent at high altitude using a right heart catheter in high-altitude pulmonary oedema resistant (HAPE-R) (24, 50) and susceptible subjects (HAPE-S) (18, 26), and mean Ppa values reported in subjects with HAPE after hospital admission (2, 21, 36, 40). The Figure illustrates that in ~ 50% of the HAPE-susceptible subjects mean Ppa exceeds the 40 mmHg mark. The horizontal bars (–) indicate median Ppa values for each group of subjects.

Among newcomers and visitors at high altitudes (> 3000 m) there are healthy individuals who present with excessive mean Ppa values, which may exceed the 40 mmHg mark. In lowlanders susceptible to HAPE mean Ppa was on average 38 mmHg with a range between 31 and 51 mmHg (26). These results are consistent with an earlier report showing in 5 HAPE susceptible subjects a mean Ppa of 39 mmHg (range 22 - 47) 24 hours after the arrival at 3100 m (18) (Figure 1). However, as shown in Figure 1 there is a considerable overlap between the mean Ppa values reported in HAPE-resistant and -susceptible individuals at high altitude. This observation may suggest that other mechanisms than pressure contribute to edema of the lung in this acute setting.

Consistently, pulmonary hemodynamic measurements at rest performed in early HAPE (26) and in all patients admitted to the hospital with HAPE (2, 21, 22, 23, 36, 40), show that left atrial pressure, as assessed by occluded (or wedged) Ppa, right atrial pressure and cardiac output are normal in HAPE. Recently, using the method of arterial occlusion, which is likely to measure pressures in vessels close to 100μm in diameter (13), we demonstrated that the pulmonary capillary pressure (Pc) is elevated in HAPE. Pc was on average 16 mmHg (range 14-18 mmHg) in HAPE-susceptible subjects without pulmonary oedema and 22 mmHg (range 20-26 mmHg) in those, who developed HAPE (26) (Figure 2). These results suggest that the Pc threshold value for edema formation in this setting is 20 mmHg. Thus, since there is evidence that the small arterioles are the site of transvascular leakage in the presence of markedly increased Ppa in hypoxia (51) and that pulmonary veins contract in response to hypoxia (39, 53) increasing the resistance downstream of the region of fluid filtration (27), it is likely that in the absence of altered pulmonary capillary permeability elevated hydrostatic pressure in the pulmonary capillary plays an important role in the pathogenesis of HAPE.

The key role of elevated Ppa in the pathogenesis of HAPE is demonstrated by the data showing that this condition is prevented or improved by the use of pulmonary vasodilators (5, 12, 34). Recent data showing that the inhalation of a beta-2-agonist at a high dose during rapid exposure to 4559 m prevented the development of HAPE cannot be taken as an argument against the role of elevated capillary pressures in the pathogenesis of HAPE (41). In fact, it is likely that the improvement of alveolar trans-epithelial transport by beta-2-agonists may shift the Pc threshold for alveolar flooding to higher Pc values by improving the equilibrium between the fluid moving across the blood-gas barrier. Moreover, although systolic Ppa was not different between the placebo and the beta-2-agonist-treated subjects in this study, one can not exclude that beta-2 stimulation at the level of the alveolar capillaries may decrease resistance in small pulmonary arteries or veins, or both.

Recent examinations of the content of alveoli in bronchoalveolar lavage (BAL) showed that both the subjects with HAPE and those who will develop HAPE within the next 24 hours presented with elevated red blood cell counts and serum-derived protein concentration, and normal alveolar macrophages and neutrophiles counts and concentration of pro-inflammatory mediators in their BAL fluid (47). Interestingly, the albumin concentration and the number of the red blood cells in the BAL fluid were significantly correlated with systolic Ppa measured by echocardiography. The threshold for albumin at a systolic Ppa was around 40 mmHg and for red blood cells around 60 mmHg (Figure 3).

In conclusion, all these recent results suggest that HAPE is a hydrostatic type of pulmonary edema; its pathophysiologic mechanism is an excessive hypoxic pulmonary vasoconstriction of small arteries and veins, which probably leads to an over-distension of the

vessel wall, which opens of the cellular junctions and possibly causes stress failure of the alveolo-capillary membrane. This suggests that signs of inflammation found in the BAL fluid of patients with advanced HAPE are a secondary event. Impairment of the alveolar transepithelial water transport and systemic inflammation may contribute to impaireds fluid homeostasis across the blood-gas barrier.

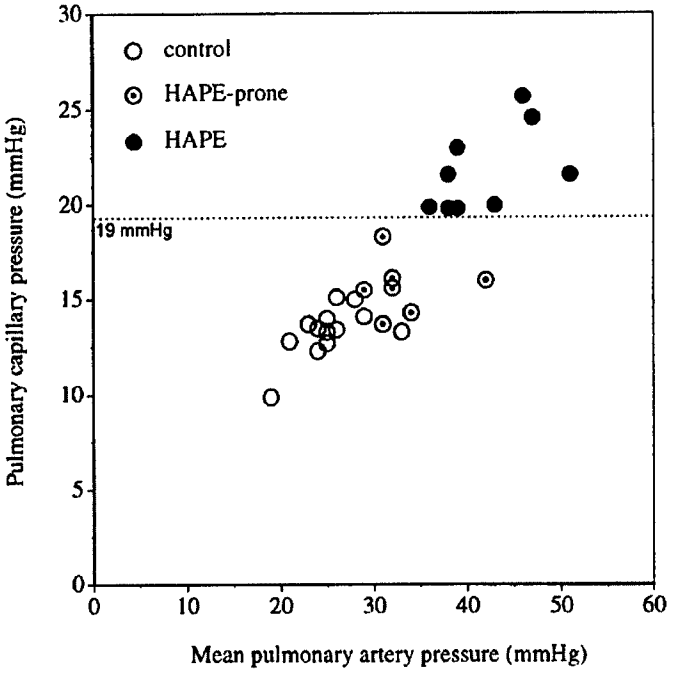

Figure 2. Relationship between individual pulmonary capillary pressure and mean pulmonary artery pressure assessed using the arterial occlusion technique, in controls (open circles), HAPE-susceptible subjects without (dotted circle) and with (closed circles) pulmonary oedema (26). The Figure shows that there is a good correlation between pulmonary capillary pressure and mean pulmonary artery pressure, and that in all subjects who develop HAPE Pc was higher than 19 mmHg. However, a cut off for mean pulmonary artery pressure could not be found.

EFFECTS OF SUBACUTE EXPOSURE TO HIGH ALTITUDE

Indirect evidence for elevated pulmonary artery pressure has been found in infants of Han descent born at low altitude, who died after an average of 2 months of residence in Lhasa (46) and in Indian soldiers, who failed to acclimatize at the very high altitude of 5800 - 6700 m (1). In infants, autopsy revealed massive hypertrophy and dilatation of the right ventricle, dilatation of the pulmonary trunk, extreme medial hypertrophy of the muscular pulmonary arteries and muscularization of the pulmonary arterioles (46). In Indian soldiers, clinical features compatible with an acute congestive right heart failure developed

between week 3 and 22, on average 11 weeks after they were stationed at altitudes between 5800 and 6700m (1). Before trekking to their post at extreme altitude, the soldiers had acclimatized during one week at 3000 m and 1 to 3 weeks at altitudes between 3000 and 4500m. After airlift to low altitude, clinical examination revealed tachypnea, tachykardia, stasis of the jugular veins, enlargement of the liver and ascites. The ECG showed right axis deviation, right ventricular hypertrophy and T-wave inversion V1 to V5-6. Chest-x-ray revealed an enlargement of the heart, prominent vascular pedicules but no pulmonary infiltrates. Echocardiography confirmed the enlargement of the right ventricle and showed normal dimensions and ejection fraction of the left ventricle. On admission, mean Ppa was on average 26 mmHg at rest and increased to 39 mmHg during mild exercise, the cardiac index increased from 3.15 to 5.28 $l.min^{-1}.m^2$. After 12-16 weeks mean Ppa decreased on average to 16 mmHg, the cardiac index was 3.5 $l.min^{-1}.m^2$. Pulmonary artery occluded pressure averaged 11 mmHg at rest, 13 mmHg during exercise and 8 mmHg after recovery. In both infants and adults, right heart failure with signs of congestion developing within weeks or months of stay at high altitude, has been called infantile and adult subacute mountain sickness, respectively. This clinical condition may be interpreted as a failure to acclimatize at high altitude.

EFFECTS OF CHRONIC EXPOSURE TO HIGH ALTITUDE

Mean resting Ppa has been reported to be lowest in Tibetans compared to Han Chinese high altitude residents and South- and North-American natives. At similar altitudes between 3658 and 3950 m, mean Ppa was on average 14 mmHg in Tibetans, 28 mmHg in Han Chinese residents of the Qinghai Province and 20 mmHg among natives of South-America (Figure 4). In Ladeville, Colorado, mean Ppa in healthy men living at 3100 m averaged 24 mmHg. Compared to Tibetans, North-Americans have a greater rise in the pulmonary artery pressure after hypoxic stimulus (10).

Transition from fetal to mature patterns of pulmonary circulation, compared to infants at sea level, in newborns at high altitude is slower and may even fail to develop. It has been reported that infants born at altitudes between 3500-4500m, may show persistent near systemic Ppa values for some time after birth (7) and that in some cases elevated mean Ppa (~ 40 mmHg) persisted during infancy (43). These observations might be linked to right heart failure in infants after birth (46) and to chronic high altitude pulmonary hypertension in adulthood (3, 8).

Congestive right heart failure associated with excessively elevated pulmonary artery pressure is described in immigrant Han Chinese after 1 to 30 years of residence at high altitude in the Himalayas (9, 35) and in high altitude natives of the Andes (16). Pulmonary hemodynamic measurements were performed in a total of 16 natives of the Andes (16), most of them performed at the altitude of 4300m, and in the 5 Han Chinese described above, who developed the disease after 11 to 36 years of stay in Lhasa (3658m) (35). Mean Ppa averaged 45 mmHg in South-Americans and 40 mmHg in Han Chinese (Figure 4). In all subjects, right atrial pressure, pulmonary artery occluded pressure (wedge pressure) and cardiac output was normal. In both populations the first symptoms associated with this condition were headache, dizziness, fatigue, insomnia and cognitive dysfunction - somnolence, slowed mental function, confusion and impaired memory. These symptoms

are the same reported in acute mountain sickness and suggest a loss of acclimatization in these individuals. Episodes suggestive for a right heart failure with dyspnea, cough, turgid jugular veins and peripheral edema follow the first symptoms within a few years in Han Chinese (9, 35), but are rare in residents of high altitude in the Andes (16). In both populations, marked cyanosis of the face and fingers, clubbing of the digits, hepatomegaly and ascites are present in the late stage of the disease. In Han Chinese and South-Americans chest radiography shows enlargement of the heart, dilatation of the pulmonary trunk and general dilatation of the small lung vessels. Kearly's B lines are in these patients characteristically absent (35). Because of excessive erythrocythosis with hematocrit levels above 70%, in South-Americans causes of death besides congestive failure of the right heart are pulmonary embolism and cerebral thrombosis. Excessive polycythemia is rare in Han Chinese. This condition in both populations has been called chronic mountain sickness.

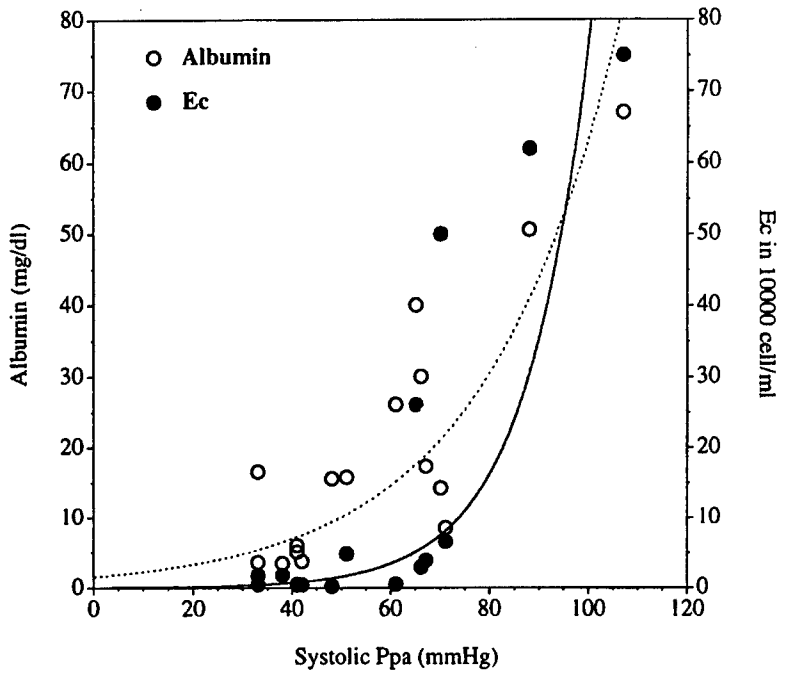

Figure 3. Individual bronchoalveolar lavage (BAL) red blood cells and albumin concentration plotted against systolic pulmonary artery pressure (sPpa) at high altitude (4559 m). The Figure shows that the threshold sPpa for the appearance in the BAL fluid of albumin was 35 mmHg and the one for red blood cells > 60 mmHg (47).

In natives of the Andes, Monge disease begins insidiously in the adult life, often during the fourth decade. Since there is some evidence that the hypoxic ventilatory response is weak in this population and that hematocrit increases with age, it has been suggested that hypoventilation leading to severe hypoxemia, hence to excessive erythrocytosis, is

the cause of Monge disease. However, there is no definite proof for that, and there are cases with excessive erythrocytosis without pulmonary hypertension (48). Moreover, in both, Han Chinese and South-Americans there is no correlation between hemoglobin or hematocrit and mean Ppa (16, 35) (Figure 5). In individuals with high altitude pulmonary hypertension (mean Ppa ~ 40 mmHg), hemoglobin concentrations range between 17 and 22 g/l in Han Chinese and between 20 and 27 g/l in the natives of the Andes. These observations suggest that excessive hypoxic pulmonary vasoconstriction rather than polycythemia-associated increased blood viscosity is the predominant cause of right heart failure in high altitude residents.

The diagnosis of Monge disease is based on the presence of its characteristic symptoms, a severe pulmonary hypertension, excessive polycythemia and a low arterial oxygen saturation for a given altitude, in the absence of other causes for a polycythemia and cyanosis (29, 30, 52). Characteristically, hematocrit values on average exceed the 70% mark (range 65-85%) and at an altitude of 4000-4500m SaO_2 is around 70%. Healthy residents at an altitude of 4000-4500m have a hematocrit around 55-60% and a SaO_2 of 80-85% (29, 30, 52). The prevalence of Monge's disease at altitudes between 4000 and 5200 m among Tibetans is 3%, Han Chinese 9.8% and Peruvians 15.6% in males and 1.6%, 6% and 8.8% in females, respectively (32).

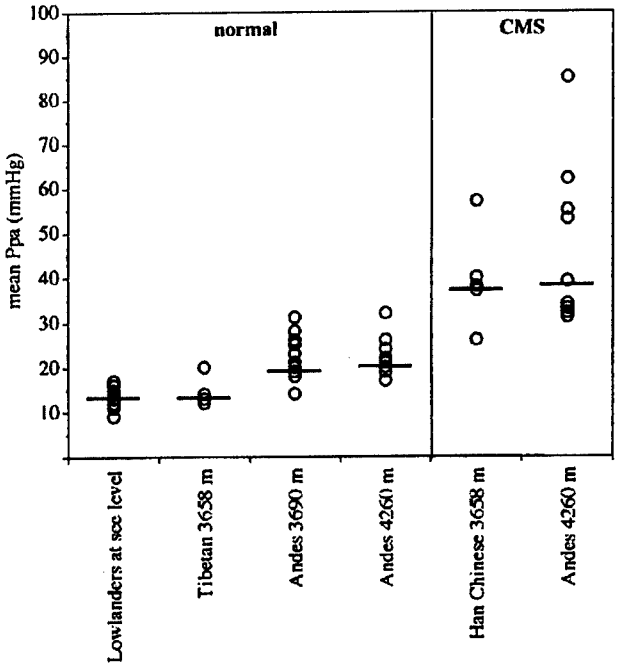

Figure 4. The Figure shows individual mean pulmonary artery pressure (Ppa) values of sea-level residents and high-altitude residents (3500 – 4300m) in the Himalayas and in South America. In the left panel we report the mean Ppa values of healthy residents (10, 19) and in the right panel values of residents with chronic right heart failure of high altitude (16, 35). The horizontal bars (–) indicate median Ppa value for each group of subjects.

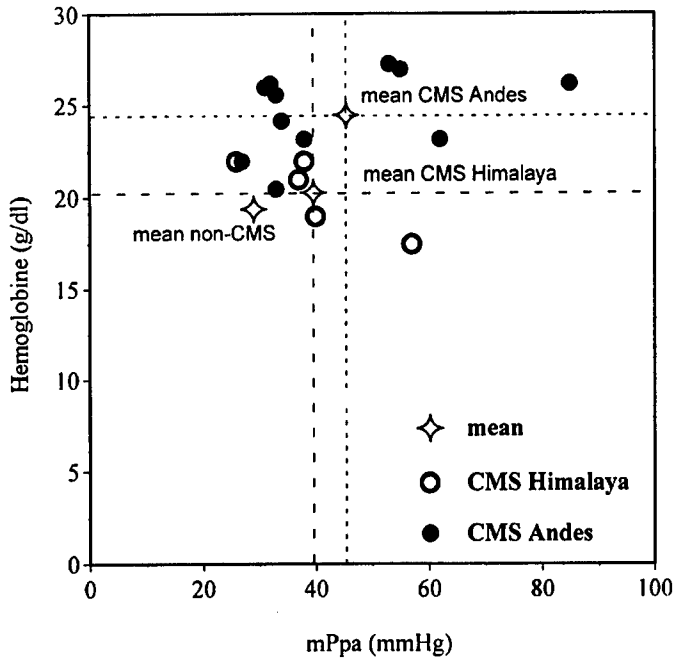

Figure 5. Relationship between haemoglobin and mean pulmonary pressure in 5 Han Chinese (35) (open circles) and 12 South-Americans (16) (closed circles) with chronic mountain sickness. Open diamonds indicate the mean values for the haemoglobin and the mean pulmonary pressure obtained for the two chronic mountain sickness populations, and those reported for high altitude residents without chronic mountain sickness living at an altitude between 3000 and 4000 m (17). The Figure shows that at comparable mean pulmonary artery pressures the hemoglobin levels in South-Americans are higher than in Han Chinese.

EXCESSIVE HYPOXIC PULMONARY VASOCONSTRICTION -THE COMMON DENOMINATOR BETWEEN ACUTE AND CHRONIC CARDIOPULMONARY DISEASE OF HIGH ALTITUDE

Pulmonary hemodynamic measurements performed in children and young adults show persistence of elevated pulmonary artery pressures at high altitude for weeks, months or years (43, 49). Histological examination of the pulmonary vessels in high altitude residents, who died from causes other than chronic mountain sickness, show persistence of the typical fetal patterns (thickened media) (3, 8). The experience of the Indian Army that its soldiers, if airlifted to extreme altitudes developed HAPE in up to 15% (44), and if acclimatised during several weeks before the stay at extreme altitude develop congestive failure of right heart failure, is highly suggestive for persistantly elevated pulmonary pressures when exposed to high altitude. Successful prevention of HAPE in spite of probably excessively elevated

pulmonary artery pressures with better acclimatisation suggests a remodelling process of the pulmonary precapillary vessels that protect the capillaries from high pressure exposure. The observations that high altitude induced changes of the pulmonary vasculature and right heart are reversible when moved to low altitude and that some high altitude residents when returning to high altitude develop HAPE (25), are further arguments in favour of a rapid adaptive process within the pulmonary vessels in front of changes in air oxygen content.

It has been shown in rats that in hypoxia, precapillary vessels of a diameter of ~ 25 μm, which normally do not have smooth muscle cells, began to generate them from adventitial fibroblasts within 24 hours (45). Light microscopic examination of nonmuscular arterioles after exposure to hypoxia show that smooth muscle began to appear by day 2 at simulated altitude, the proportion of muscularised arterioles increasing along with increasing Ppa (37, 38). Interestingly, all these studies show that after return to normoxia, smooth muscle cells persisted in normally not muscularized arterioles, suggesting that smooth muscle cells may remain for very long time after chronic exposure to hypoxia. If applicable to humans, these findings suggest that excessive hypoxic pulmonary vasoconstriction stays at the origin of both HAPE and high altitude associated right heart failure. Furthermore, these findings in rats also suggest that structural remodelling of the precapillary pulmonary vessels is probably crucial for the protection of the pulmonary capillaries from excessively elevated pulmonary artery pressures and hence for the prevention of HAPE. However, muscularisation of normally non-muscular pulmonary vessels may additionally increase Ppa during chronic high altitude exposure leading finally to right heart failure.

TERMINOLOGY

Carlos Monge and coworkers originally used the name subacute mountain sickness to describe the persistence of headache, anorexia, nausea, dizziness and difficulty to sleep (usual symptoms of acute mountain sickness) during weeks and months after arrival at high altitude. (29, 31). Typically, in all these patients, mainly mining workers of the Peruvian Andes, physical findings suggesting congestive failure of the right heart, were absent. Unfortunately, the name "subacute mountain sickness" has recently also been used to describe the rapid - within a few weeks or months after ascent to high altitude - development of congestive right heart failure in Han Chinese infants and Indian soldiers (1, 46). We think that the name "subacute mountains sickness" should be reserved for the description of the original syndromes published 1937, and the name "acute right heart failure of high altitude" should be used to describe the syndrome described by Anand and coworkers. Accordingly, congestive failure of the right heart observed in immigrants born at low altitude after years of residence at high altitude and in residents of high altitude should be called "chronic right heart failure of high altitude". The name "chronic mountain sickness" should be used to summarize unspecific symptoms preceding the development of congestive right heart failure in the absence of polycythemia. Finally, the name "Monge disease" should be reserved exclusively for those high altitude residents, who present with the trias pulmonary hypertension, excessive erythrocytosis and severe hypoxemia (Table 1).

Table 1. Proposal for a new terminology of high altitude pulmonary hypertension related diseases

High Altitude Pulmonary Hypertension		
Exposure time	*Old nomenclature*	*New nomenclature*
Acute (2-5 days)	High altitude pulmonary edema	**High altitude pulmonary edema**
Subacute (1-4 weeks)	Infantile/Adult subacute mountain sickness	**Acute right heart failure of high altitude**
Chronic (> 1 year)	Chronic mountain sickness or Monge disease (Hemoglobin $\leq$ 21 g/dl)	**Chronic right heart failure of high altitude** without excessive erythrocythosis (hemoglobin < 21 g/dl)
	Chronic mountain sickness with Monge disease (Hemoglobin > 21 g/dl)	**Chronic right heart failure of high altitude with excessive erythrocythosis** (hemoglobin $\geq$ 21 g/dl) or Monge disease

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Chapter 14

OXIDATIVE STRESS AND AGING

Wulf Dröge

Abstract: Free radical-derived reactive oxygen species (ROS) are constantly generated in most living tissue and can potentially damage DNA, proteins and lipids. "Oxidative stress" occurs if ROS reach abnormally high concentrations. Harman was the first to propose that the damaging effects of ROS may play a key role in the mechanism of aging. Genetic studies of such distantly related species as *C. elegans*, *Drosophila melanogaster*, and mice support this hypothesis. However, ROS are not only a cause of structural damage, but also physiologically important mediators in biological signaling processes. Abnormally high levels of ROS may therefore lead to dysregulation of redox-sensitive signaling pathways. The redox-sensitive targets in these pathways are often signaling proteins with redox-sensitive cysteine residues which are oxidized to sulfenic acid moieties and mixed disulfides, thereby altering the signaling function of the protein. Because the formation of these mixed disulfides can also occur through a prooxidative shift in the intracellular thiol/disulfide redox status (REDST), the respective signaling pathways respond not only to ROS but also to changes in REDST. Information about the concentration of ROS in living tissue is scarce, but aging-related changes in REDST are well documented. Several studies with cell cultures or experimental animals have shown that the oxidative shift in the *intracellular* glutathione REDST is typically associated with cellular dysfunction. Complementary studies in humans have shown that oxidative changes in the plasma (*i.e., extracellular*) REDST are correlated with aging-related pathophysiological processes. The available evidence suggests that these changes play a key role in various conditions which limit the human life span. Several attempts have been made to ameliorate the consequences of aging by thiol-containing antioxidants, but this approach requires a detailed knowledge of the effects of thiol-containing antioxidants on cysteine homeostasis, REDST, and redox-sensitive signaling pathways of the host.

Key Words: reactive oxygen species (ROS), thiol/disulfide redox status, glutathione, redox regulation

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INTRODUCTION: THE POSITIVE AND NEGATIVE FUNCTIONS OF REACTIVE OXYGEN SPECIES (ROS)

At a conference on hypoxia, it may be most appropriate to start with a special reference to ischemia and reperfusion which are known to cause oxidative stress and to account for tissue injury and serious complications in organ transplantation, myocardial infarction and stroke (18,19). The oxidative stress is due to the high concentration of superoxide anion radicals (1,9,12,13,25,32,33,35,41,42,55) which are generated by xanthine oxidase (20), and NAD(P)H oxidase (43).

In view of the highly toxic effect of superoxide and related reactive oxygen species (ROS) in ischemia and reperfusion and other conditions of oxidative stress, it is important to note, however, that superoxide radicals and certain ROS are constantly generated in most cells and living tissues and mediate important positive physiological functions (reviewed in ref. 14). At physiological concentrations, superoxide and superoxide-derived ROS are known to a play a key role as effectors in the immunological defense against pathogens and as regulatory mediators in the signaling processes. The protective effects against pathogens are mainly explained by the aggressive chemical nature of ROS. The regulation of physiological processes is essentially mediated by interaction of ROS with redox-sensitive proteins in signaling cascades (reviewed in ref. 14).

Whereas higher life would not be possible without the participation of ROS in various physiological functions, abnormally high concentrations of ROS cause "oxidative stress". The term "oxidative stress" is commonly associated with radical-inflicted oxidative damage of DNA, proteins, and lipids. However, in view of the regulatory role of ROS in numerous signaling cascades, it is not surprising that excessive concentrations of ROS also cause dysregulations of signaling processes, which eventually may lead to altered gene expression.

The free radical theory of aging which was proposed by Harman almost 50 years ago (22) has originally been dealing with the damaging effects of free radicals on the structural components of cells and tissues. Today, this theory is still alive but has to be extended to include the role of oxidative stress in the dysregulation of gene expression.

THE ROLE OF ROS IN REDOX REGULATION

The terms "redox regulation" or "redox signaling" are widely used to describe regulatory processes in which the signal is delivered through redox chemistry. Redox signaling is used by a wide range of cells and organisms (reviewed in ref. 14). Whenever physiological processes are controlled by redox regulation through free radicals, these radicals are typically generated by tightly regulated enzymes. Specifically, three isoforms of nitric oxide synthase form the nitric oxide radical from molecular oxygen and the amino acid arginine (44), and the various NADPH oxidase isoforms generate superoxide radicals from molecular oxygen (reviewed in ref. 14). In addition, ROS are also generated at an uncontrolled rate as side-products of other metabolic processes. The quantitatively most important source of superoxide radicals in living tissue is the mitochondrial electron transport chain (8,10,39,56).

That superoxide radicals may not only account for damaging effects, as previously thought, but also play a positive role in the regulation of gene expression was first observed in immunological experiments in 1987. In studies of purified T cells we found that the production of the T cell growth factor interleukin-2 was strongly enhanced by superoxide and hydrogen peroxide (46). The more detailed investigation of the oxidative enhancement of lymphocyte functions revealed a remarkable redundance of redox-sensitive signaling molecules (reviewed in ref. 14). Protein tyrosine kinases (PTKs) are known to play a major role in the processing of the receptor-mediated signals after antigenic stimulation. Hydrogen peroxide can enhance these signaling processes by directly activating certain PTKs in these pathways or by inactivating protein tyrosine phosphatases (PTPs), which negatively regulate the effects of PTKs. By inactivating the inhibitor, this effect contributes to the oxidative enhancement of lymphocyte functions (reviewed in ref. 14). In the presence of sufficiently large concentrations of antigen, lymphocytes can be readily stimulated in the absence of an oxidative microenvironment. Under more physiological conditions, however, i.e., after infection with a small number of pathogens, the production of hydrogen peroxide by activated macrophages and neutrophils in the inflammatory environment is expected to enhance the signaling cascade and to allow T cells to respond to much lower antigen concentrations. Under certain conditions, this mechanism may be life-saving.

Other cell surface receptors, such as the EGF-receptor and the insulin receptor, do not rely on ROS produced by other cells in the microenvironment but trigger intracellularly the production of superoxide and hydrogen peroxide and enhance thereby their own signaling cascade. EGF, for example, was shown to stimulate NA(D)PH oxidase (3), and hydrogen peroxide, in turn, was shown to enhance EGF receptor-mediated signaling (57,14).

The transcription factors AP-1 and NF-κB are the best investigated redox-responsive transcription factors. The transcription factor AP-1 is typically composed of c-Fos and c-Jun proteins and involved in various differentiation processes. In T lymphocytes AP-1 regulates the expression of the interleukin-2 gene and other immunologically relevant genes. The oxidative activation of AP-1 activity is based on the oxidative activation of Jun-N-terminal kinase (JNK) (59), i.e., a mitogen-activated protein kinase (MAPK), which phosphorylates the serine residues 63 and 73 of the NH_2-terminal transactivation domain of cJun, a domain which is required for functional activation of this transcription factor (reviewed in ref. 14).

The transcription factor NF-κB is involved in a wide variety of biological responses, including inflammatory reactions and the induced expression of the interleukin-2 gene. NF-κB was the first eukaryotic transcription factor shown to directly respond to oxidative stress in certain types of cells (49). Accordingly, NF-κB is inhibited by antioxidants, such as cysteine (36,38,47,51,52). The available evidence suggests that the oxidative activation of NF-κB is mediated by at least two different mechanisms. The first one involves oxidative degradation of the inhibitory protein IκB. A second mechanism involves the oxidative enhancement of the upstream signaling cascade (48,23).

Expectedly, the redox-responsive transcription factors NF-κB and AP-1 also play a role in oxidative stress. The abnormal activation of the MAPKs JNK and p38, and of the transcription factors NF-κB and AP-1 in ischemia and reperfusion injury is an example of the dysregulation of signaling processes by oxidative stress (11,28,34) and accounts for inflammatory and apoptotic processes in these clinical conditions (26,50).

MANY REDOX-SENSITIVE SIGNALING PATHWAYS RESPOND TO CHANGES IN THE CELLULAR THIOL/DISULFIDE REDOX STATUS (REDST)

In several cases, changes in the intracellular REDST have been shown to trigger the same redox-responsive signaling pathways which normally are triggered by hydrogen peroxide (17,23,27). The activation of AP-1 and its upstream signaling cascades, for example, was shown to be enhanced by a moderate pro-oxidative shift in the intracellular glutathione REDST (17,23). A similar change in redox status was also shown to activate the transcription factor NF-κB through the phosphorylation of the inhibitory protein IκB-α and the activation of the IκB kinase IKKα in T cells (23).

Using the glutathione reductase inhibitor BCNU, we have been able to show the effect of REDST on the immunologically important transcription factor AP-1 in lymphocytes. Concentrations of BCNU between 10 and 100 μM cause a substantial increase in the intracellular glutathione disulfide concentration and a corresponding decrease in reduced glutathione. Under these conditions, the transcription factor AP-1 is stimulated more than 10-fold as detected by the expression of a chloramphenicol acetyltransferase (CAT) reporter construct under the control of six AP-1 binding sites (17).

The activation of the transcription factor AP-1 is under the control of the MAPK JNK. Stimulation of lymphocytes by anti-CD3- and anti-CD28-antibodies, which are directed against the T cell receptor and the costimulatory CD28-receptor, respectively, causes by itself the production of hydrogen peroxide (31) and a substantial oxidative shift in the glutathione REDST, which is further enhanced by the addition of either BCNU or hydrogen peroxide (23). The analysis of the MAPKs JNK and p38 revealed that anti-CD3- and anti-CD28-antibodies cause by themselves a moderate phosphorylation of cJun and p38 MAPK, which is synergistically enhanced by either BCNU or by hydrogen peroxide. BCNU and hydrogen peroxide cause by themselves only a moderate activation of these signaling proteins (23).

The list of signaling mechanisms known to respond to changes in the thiol/disulfide redox status has been growing in recent years and includes amongst other systems the bacterial OxyR, the insulin receptor kinase activity, Src family kinases, PTPs, JNK, and p38 MAPK signaling pathways, the transcription factors AP-1 and NF-κB, the amplification of immunological functions, and signaling in replicative senescence (reviewed in ref. 14).

The influence of the REDST on signaling processes has been studied at the molecular level in the cases of PTPs and the bacterial OxyR system. The oxidative inhibition of PTPs was shown to involve a redox-sensitive cysteine moiety in the catalytic site (4-6). The oxidative conversion of cysteine into sulfenic acid renders the enzyme inactive. The chemically highly reactive sulfenic acid moiety, in turn, interacts spontaneously with intracellular glutathione to yield a mixed protein-glutathione disulfide which is also enzymatically inactive. The active PTP is eventually regenerated by reduction of the disulfide by another glutathione molecule. Since this reaction is reversible, it is to be expected that the active PTP can be inactivated by glutathione disulfide and that the PTP activity is sensitive to the REDST of the cell.

The bacterial OxyR system regulates the expression of various protective enzymes in response to oxidative stress. The OxyR protein is typically present in bacteria in the inac-

tive, reduced form which expresses reactive thiol groups. After interaction with hydrogen peroxide, these thiols are converted into disulfides and thereby activate the OxyR protein which signals the expression of genes for protective enzymes (7,53,61). Alternatively, the oxidative activation of this protein is also mediated by an oxidative shift in REDST (2). In contrast to the oxidative inactivation of PTP, the oxidative activation of OxyR is an example of an oxidatively induced gain of function.

AGE-RELATED CHANGES IN THE PLASMA REDST

ROS production is difficult to measure in biological tissues. In most cases, the investigators have only been able to document the effects of oxidative stress, such as increased levels of lipid peroxidation, DNA oxidation and protein oxidation (reviewed in ref. 14). Since loss of skeletal muscle mass is one of the hallmarks of age-related wasting, the massive age-related manifestations of oxidative damage which were found in skeletal muscle tissue of rhesus macaques may be of special interest (60). In studies of rats, it was shown that muscle fibers harboring mitochondrial deletions often display increased steady state levels of oxidative nucleic damage (58). Unfortunately, however, these indirect data do not allow us to distinguish whether these age-related changes may result from an age-related increase in ROS production per time unit or from a decreased clearance of oxidative damage.

Compared to the difficulties in measuring ROS production *in vivo*, changes in the REDST can be demonstrated more easily. Certain methods can even be applied to larger clinical trials. In a study of more than 200 healthy human subjects we have been able to demonstrate a substantial age-related oxidative shift of the plasma redox status, and preliminary evidence suggest that changes in this order of magnitude have indeed a significant effect on certain redox-sensitive signaling processes. Originally, we have demonstrated this shift by the increase in the plasma concentration of oxidized cysteine (cystine) and the simultaneous decrease in the plasma thiol concentration which represents mainly reduced cysteine (21). Since cystine is formed by oxidation from two cysteine molecules, the REDST was computed as the square of the thiol concentration devided by the concentration of cystine. The regression function of the REDST vs. age indicates that the REDST decreases approximately 4-fold between the third and the nineth decade of life. This oxidative shift in plasma REDST has been confirmed in the meantime by other laboratories with respect to other thiol/disulfide redox couples and by different experimental methods.

The decrease in the plasma cysteine level and REDST has major biochemical consequences. Albumin, for example, (i.e. the quantitatively most important redox-sensitive plasma protein) shows an increase in its oxidized forms and a concomitant age-related decrease in the total plasma albumin concentration (reviewed in ref. 21). Moreover, since most somatic cells have a very weak or no transport activity for the relatively larger amino acid cystine, they are strongly dependent on the availability of reduced cysteine in the extracellular environment (reviewed in ref. 16). The age-related decrease in the plasma thiol (cysteine) concentration is thus likely to account for the age-related decrease in intracellular glutathione concentration and to some extent for the age-related decrease in protein synthesis in these cells and tissues. Several studies with cell cultures or experimental animals have shown that the decrease in *intracellular* glutathione and/or the oxidative shift in

REDST are typically associated with cellular dysfunction.

In contrast to studies of human subjects, experimental animal studies have the advantage that aging-related changes can be readily characterized at the intracellular level. In animal studies an age-related decrease in intracellular glutathione concentration and/or REDST has been demonstrated in whole blood, peripheral blood mononuclear cells, skeletal muscle tissue, liver, kidney, brain, and in retinal glia cells (reviewed in ref. 16). These changes were found to be associated with various manifestations of oxidative stress, including cellular dysfunction, mitochondrial decay, and the impairment of cognitive functions.

In studies on human subjects, an age-related decrease in the intracellular glutathione concentration and/or REDST has been demonstrated in whole blood, peripheral blood mononuclear cells and the skeletal muscle tissue (reviewed in ref. 16). An age-related decrease in cysteine (thiol) and REDST has been demonstrated in the plasma. Such changes in plasma REDST were found to be correlated with aging-related degenerative processes, conditions and diseases which may determine the human life span. These include the loss of muscle mass and muscle function (wasting), cardiovascular diseases, malignant diseases, and the decrease in the plasma albumin concentration (reviewed in refs. 15,16).

GENETIC EVIDENCE LINKING OXIDATIVE STRESS TO LIFE SPAN

Genetic evidence linking oxidative stress to life span has been obtained for different animal species. In *Caenorhabditis elegans,* the *daf-2* mutation causes longevity by increasing manganese superoxide dismutase (Mn-SOD) expression (24). Catalase is required to extend the life span in *daf-C* and *clk-1* mutants of *C.elegans* (54). *Drosophila* strains with extracopies of genes encoding SOD and catalase live longer (40,45). Also, the mth mutant of *Drosophila* was found to live longer and has increased resistance to a free radical generator (30). Mice carrying the mutation $p66^{shc}$ were found to have an increased life span associated with increased resistance to oxidative stress (37); and another study of mice showed that aging is associated with increased transcription of oxidative stress-inducible genes (29).

CONCLUSIONS

Taken together, the available evidence supports strongly the hypothesis that the process of aging is, at least to a large extent, the consequence of oxidative stress and an oxidative shift in REDST. These oxidative processes may cause not only oxidative damage of cellular structures but, perhaps more importantly, the dysregulation of redox-sensitive signaling cascades and gene expression. The tripeptide glutathione is the most important intracellular thiol antioxidant, and there is a growing body of evidence for an age-related decrease in glutathione concentration and an oxidative shift in glutathione REDST in most cell types tested so far. Animal studies have been very useful to demonstrate the linkage between changes in intracellular glutathione level or REDST and cellular manifestations of oxidative stress. The specific merits of the studies of humans have been that they have

demonstrated a linkage between the age-related oxidative shift in plasma REDST and various age-related diseases and conditions which limit the human life span. Thiol antioxidants were found to ameliorate various aging-related processes, but this approach requires a detailed knowledge of the effects of thiol-containing antioxidants on cysteine homeostasis, REDST, and redox-sensitive signaling pathways of the host. Obviously, thiol-containing drugs or dietary supplements ought to be used with caution.

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Chapter 15

RADICAL DIOXYGEN:
From gas to (unpaired!) electrons

Damian Miles Bailey

Abstract: Photosynthesising cyanobacteria breathed life into what was 1000 million years ago considered a reductive atmosphere, thus providing a selective pressure for the evolution of O_2-dependent organisms. However, the fact that molecular O_2 exists in air as a free radical renders it a double-edged sword, capable of sustaining life in physiologically controlled amounts, yet fatal when in excess. The controlled delivery and stepwise reduction in PO_2 from air to mitochondrion may in itself be considered an evolutionary antioxidant to cope with this biological conundrum. The present review will discuss the potential roles, both good and bad, for free radicals during human adaptation to altered environmental PO_2. By combining electron paramagnetic resonance spectroscopy with spin-trapping, we provide direct molecular evidence for increased O_2 and carbon-centered radical generation at high-altitude which may seem paradoxical in light of the reduced PO_2. Radical-mediated contributions to tissue damage and their subsequent role in the pathogenesis of AMS, HAPE and HACE will also be critically examined. Finally, we focus on the sources, mechanisms and functional significance of free radical generation in hypoxia, with a brief consideration of their more positive role as putative signal transductants, capable of adjusting cellular homeostasis and initiating protective adaptation. Our preliminary studies in humans suggest that radical generation by skeletal muscle is exquisitely sensitive to intracellular PO_2 which may provide a unifying theory to explain the "free radical paradox" of high-altitude.

Key Words: hypoxia, antioxidants, EPR spectroscopy, spin-trapping, mitochondria, oxygen-sensing.

OXYGEN: PARADOX OF THE PANACEA AND POISON

Our continued fascination with the element oxygen (O_2), first discovered by Joseph Priestley (1733-1804), is eminently justified for without it we would simply not survive.

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

Maintenance of an "adequate" supply of molecular O_2 to respiring mammalian cells is of evolutionary significance because it serves as the terminal electron acceptor in mitochondrial oxidative phosphorylation and several enzymatic processes require O_2 as a substrate. Photosynthesising cyanobacteria constitute the oldest fossils on record (23) and have breathed life into what was 1000 million years ago considered to be a reductive atmosphere containing only 1-2% O_2 (1). Contemporary estimates now suggest that the green plants on earth combine a total of 150 billion tons of carbon (from CO_2) with 25 billion tons of H_2 (from H_2O) to liberate 400 billion tons of O_2 each year. This accounts for the present day atmospheric content of O_2, which has persisted for the last one tenth of Earth's existence (Figure 1).

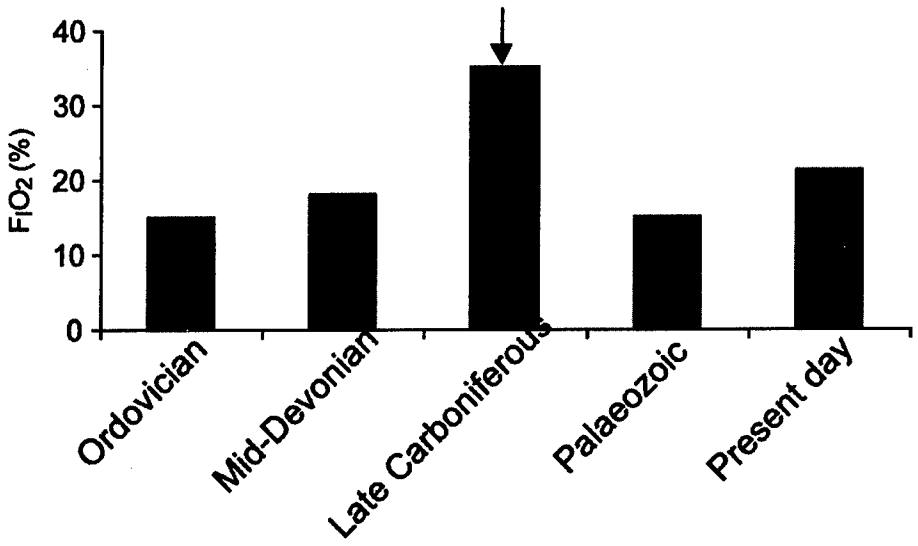


Figure 1. Fluctuations in the palaeoatmospheric O_2 concentration during the Earth's history. Digits are expressed in millions of years ago relative to the present day. Calculations based on the exchange rate of fixed carbon between the atmosphere, ocean and sediments as described by Graham *et al.* (12). The hyperoxic environment associated with the late Carboniferous period (indicated by the arrow), may have "triggered" evolution of antioxidant defences to cope with this "O_2 excess."

However, close examination of the sub-atomic structure of O_2 reveals a more nebulous side to a gas traditionally considered the elixir of life. Though capable of sustaining life in physiologically controlled amounts, this "double-edged sword," can prove paradoxically fatal when in excess. The diatomic O_2 molecule exists in air as a free radical due to the existence of two unpaired electrons located in different antibonding orbitals. However, unlike the majority of free radical species, the quantum mechanics of O_2, in particular its spin restriction, render its biological reactivity comparatively weak. This is somewhat fortunate from an evolutionary point of view and may explain why the complex organic compounds of the human body do not simply combust on direct exposure to air! Furthermore, it has been suggested that during the tetravalent reduction of O_2 to H_2O, some of the

electrons that flux through the mitochondrial electron transport chain can "leak" directly on to O_2 generating the univalent reductant, superoxide. This radical is considered potentially cytotoxic *in vivo* and in conjunction with other intermediates, is capable of initiating and propogating cellular membrane destabilization and damage. Though principally providing a "pressure-head" to maintain O_2 flux from air to mitochondrion, the *in-vivo* resistances offered to O_2 delivery may therefore have evolved, by chance or by fate, to protect the cell from the full force of its mutagenic effects, a surrogate antioxidant defence system!

Decreased O_2 availability such as that typically experienced during exposure to terrestrial high-altitude would therefore be expected to attenuate potentially damaging radical reactions. However, preliminary findings discussed in the present chapter suggest the reverse, implying that the present day "normoxic O_2-milieu" provides man with the optimal "redox state", a physiological equilibrium between pathological reactions associated with O_2 excess *vs.* O_2 lack.

In light of the fundamental principles described here and the previous chapter outlined by Dr. Dröge, the present discussion will focus on the potential roles, both good and bad, of free radicals during human adaptation to terrestrial high-altitude. I will briefly address some of the technical challenges associated with the measurement of these elusive biomolecules and introduce the novel concept of electron paramagnetic resonance (EPR) spectroscopy and its application to high-altitude. Radical-mediated contributions to tissue damage and their potential role in the pathogenesis of altitude illness and associated physiological sequelae will also be critically examined. Finally, I will describe some preliminary laboratory-based studies that may help identify the source and potential mechanisms associated with free radical generation at high-altitude; a phenomenon that at first glance, may prove somewhat of a paradox!

MOLECULAR DETECTION OF FREE RADICALS AT HIGH-ALTITUDE

The examination of free radical species in biological materials remains a formidable analytical challenge due primarily to their high reactivity and low steady-state concentration (10). Consequently, investigators have typically relied on non-specific markers, formed as a consequence of the molecular interaction of free radicals with cellular components containing lipids and proteins, an indirect approach referred to as "footprinting".

However, the advent of spin trapping has helped overcome some of these limitations, and has permitted the direct detection of free radicals in humans. The basis of this approach involves an *ex-vivo* reaction of a diamagnetic spin trap with a highly reactive paramagnetic free radical which yields a resonance stabilized adduct that can subsequently be detected via EPR spectroscopy (9). EPR detection relies on the physical behaviour of the dipole associated with the unpaired electron subsequent to application of a constant microwave frequency and varied external magnetic field (27).

In combination with indirect biomarkers of free radical-mediated lipid peroxidation, we have applied the combined techniques of spin trapping using the nitrone, α-phenyl-*tert*-butylnitrone (PBN/$C_{11}H_{15}NO$) and EPR spectroscopy to examine potential changes in free radical generation following ascent to high-altitude and following prophylactic antioxidant supplementation.

Radicals and Antioxidants

Following ethical approval, sixteen healthy males participated in a randomized double-blind placebo-controlled trial. Eight subjects were instructed to ingest a combination of water and fat soluble antioxidant vitamins (daily bolus dose of 1000mg *L*-ascorbic acid, 400 *iu* of *dl*-α-tocopherol acetate and 600mg of α-lipoic acid) and the remaining eight subjects ingested a placebo. Supplementation was initiated at sea-level, seven days prior to departure to India, for four days in Delhi and during a seven day day ascent to 4,780m.

Species Detected

Compared to sea-level control conditions, antioxidants decreased EPR spectral amplitude at high-altitude whereas an increase was observed in the placebo group (Figure 2). Figure 3 provides qualitative examples of typical spectra obtained. The spin trap contains a hydrogen atom beta (β) that interacts with the unpaired electron causing each of the molecule's three nitrogen lines to be split into doublets resulting in the characteristic six-line spectrum, or "triplet-of-doublets", the molecular signature of the nitroxide spin adduct (R_2NO). Close examination of the nuclear hyperfine splitting of the PBN adduct reveals some interesting information about the species of radical(s) trapped. The splitting of two resonance lines (defined as the nitrogen (a^N) and hydrogen (a_β^H) splittings as illustrated in Figure 3) originates from the interaction of the electron spin with molecular magnetic nuclei.

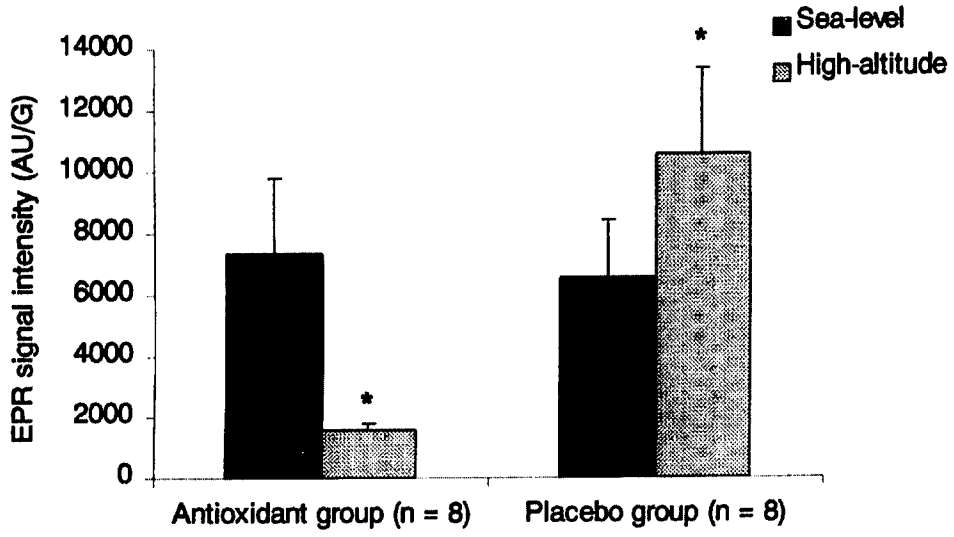


Figure 2. Quantitative changes in the resting EPR spectral signal intensity of the PBN adduct at high-altitude and following prophylactic antioxidant vitamin supplementation. Values are mean ± SD and expressed in AU per Gauss. Main effects for location (sea-level *vs.* high-altitude) and group (antioxidant *vs.* placebo) and interaction observed ($P < 0.05$). * indicates difference within and between groups ($P < 0.05$).

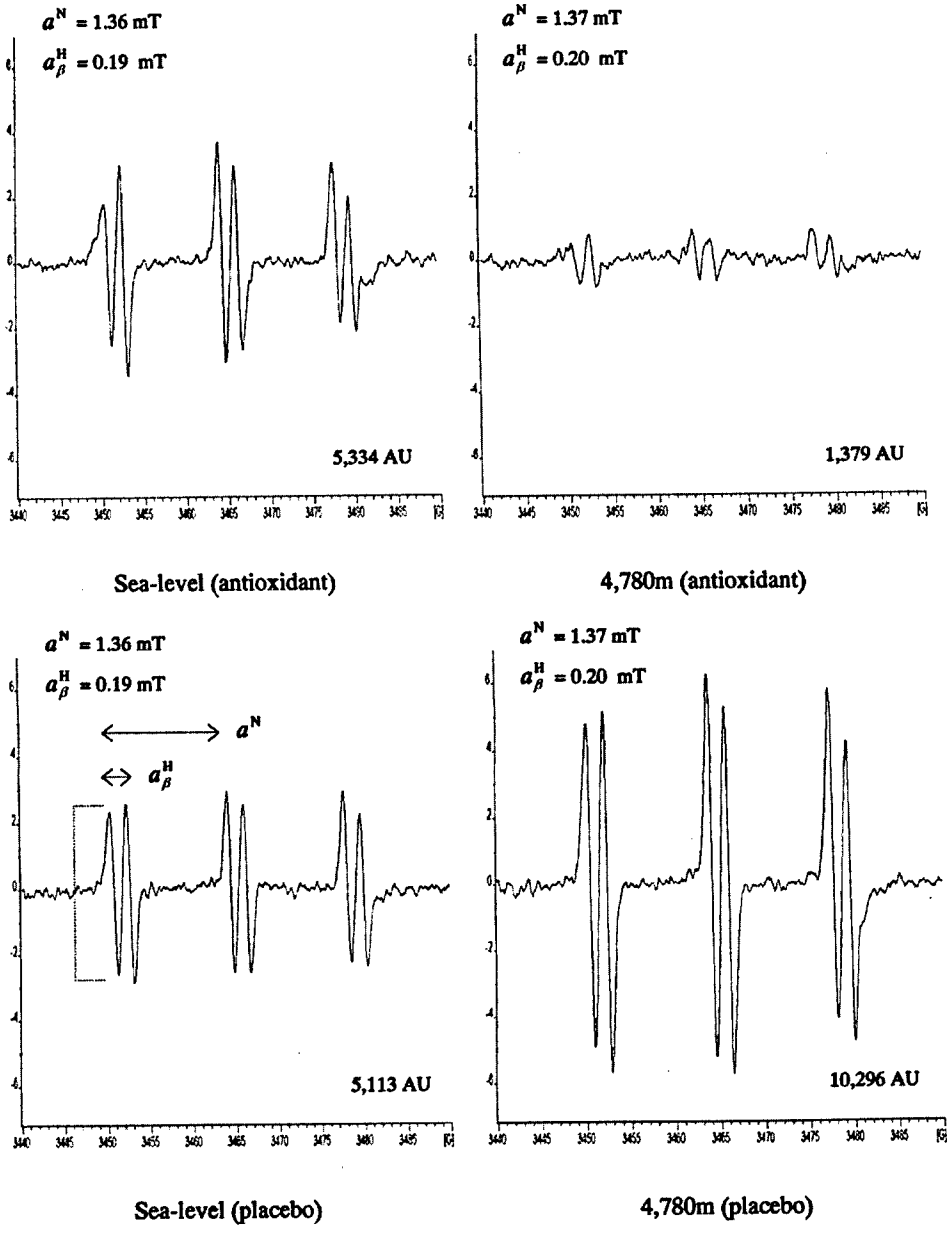


Figure 3. Typical changes in the electron paramagnetic resonance (EPR) spectroscopic signal intensity of venous α-phenyl-*tert*-butylnitrone (PBN) spin adducts following antioxidant prophylaxis at sea-level and high-altitude ($(n = 2)$: 1 subject for placebo and 1 subject for antioxidant supplementation). Coupling constants for the nitrogen (a^N) and hydrogen (a_β^H) splittings and relative spin concentration (calculated as the mean of each of the peaks) are also displayed. Note that sea-level blood sampling was conducted prior to the start of supplementation.

The splittings are consistent with the trapping of either a carbon-centered species such as the alkyl radical and/or an O_2-centered alkoxyl or peroxyl radical. The trapping of PBN-peroxyl radicals is quite unlikely however, despite a comparatively long half-life ($T_{1/2}$) of 7 s (19), since they typically display smaller splitting constants (a^N= 1.35 mT and a_β^H = 0.14 mT) and are unstable at room temperature (17). In contrast, the PBN-alkoxyl radical is comparatively more stable and may prove the predominant species detected using this technique. However, intermediate values for the coupling constants and the clear assymmetry of each triplet of doublets indicates the presence of several radical adducts (personal communication, Dr. CC Rowlands), which remain to be fully resolved.

If we are indeed trapping the alkoxyl radical with a $T_{1/2}$ as low as 10^{-6} s (19), then we are clearly detecting species formed distal to the instrumented vasculature. To put this into perspective, if the original alkoxyl radical was generated in the vasculature and was in contact with the bevel of the needle, less than 1% of its original concentration would be detectable by the time it had travelled less than 0.5% of the total distance between needle and spin trap (assuming a venous blood velocity of 5m/s and distance between needle-tip and spin trap of 10cm).

Therefore, we postulate that the signals retrieved with this approach may reflect the oxidation products of a continuous cascade involving the metal-catalyzed decomposition of lipid hydroperoxides originating from primary radical-mediated damage to membrane phospholipids *in-vivo*. Recent research conducted in our laboratories adds some support to this contention. We have consistently demonstrated a concomittant increase in venous lipid hydroperoxides, one of the major, initial reactants of lipid peroxidation, and EPR signal intensity of the PBN adduct (4, 5). Furthermore, *in-vitro* oxidation of the polyunsaturated fatty acids, linoleic (19:2) and α-linolenic acid (19:3) yield identical coupling constants (a^N = 1.38 mT, a_β^H= 0.17-0.18 mT) to those observed in the present study (GW Davison, unpublished observations). Thus, while further research is clearly required to unequivocally identify the species of radical trapped, we are confident that the *ex-vivo* technique employed in the present study represents oxidative events that principally occur *in-vivo*. A brief pictorial overview of the proposed reactions including "candidate" species that constitute the initiating, propagating and decomposition cascades are presented in Figure 4.

FREE RADICALS, TISSUE DAMAGE AND ALTITUDE-ILLNESS

The Challenge

A thorough examination of the mechanisms considered important in the etiology of high-altitude illness is beyond the scope of the present review. In brief, attention has focused on the physiological forces that promote edema, arguably the major determinant factor responsible for associated symptoms. However, a great deal of emphasis has traditionally been placed on hydrostatic factors, to the expense of potential alterations in membrane permeability which may compound any vascular leak.

The histological constitution of the vasculature considered major loci of the "altitude edemas" renders it especially prone to free radical-mediated oxidative stress as illustrated in Figure 5. An excessive generation of free radicals has subsequently been associated with tissue injury characteristic of most, if not all, clinical pathologies, notably pulmonary dis-

ease such as the adult respiratory distress syndrome and a variety of central nervous system disorders caused by neurodegeneration, ischemia or trauma (13). However, there is insufficient evidence at present to incriminate radicals themselves as primary initiators of tissue damage and thus by consequence, disease; they may purely prove an epiphenomenon, a possibility that is the source of much frustration amongst many a free radical biologist! Our uncertainties are based on an inherent reliance on non-specific biomarkers, failure to examine the illness early in its evolution, differences in the histological constitution of associated vasculature and difficulties encountered when attempting to disassociate hemodynamic from permeability phenomena. However, preliminary findings from our laboratory tentatively suggest at least a contributory role for oxidative tissue damage.

Supporting Evidence and Emerging Findings

AMS and Infection; a Common Pathophysiology?

During the 1998 British Mt. Kanchenjunga medical expedition, a comparatively greater increase in free radical-mediated biomarkers of lipid peroxidation and sarcolemmal membrane permeability was observed in subjects diagnozed with clinical AMS and infection at 5100m (5). While the "acute form" of AMS is classically neurogenic and infection microbial in origin, the fact that both states exhibited similar, non-specific symptoms, may prove a complicating factor when attempting to diagnoze and treat altitude-related illness, at least during a prolonged ascent to terrestrial high-altitude.

Peripheral biomarkers representative of increased free radical-mediated skeletal muscle damage and amino acids known to depress immune function (18) were selectively altered in the AMS and infected states compared to apparently healthy control states (7). These findings, while not establishing cause-and-effect, tentatively suggest that free radical-mediated damage to skeletal muscle may alter the peripheral release of immunostimulatory amino acids increasing susceptibility to and/or delaying recovery from opportunistic infections and thus by consequence, AMS. An overview of related symptoms and potential mechanisms common to both states is illustrated in Figure 6.

Defining a Temporal Association Between Tissue Damage and Physical Symptoms

It should be noted that an acute examination of molecular markers of tissue damage concurrent with established altitude illness does not establish cause from effect. Furthermore, the transient release of intracellular myofiber proteins or inflammatory markers into the peripheral circulation subsequent to ultrastructural damage may be inconsistent with the more acute onset of associated physical sequelae; a clear limitation when attempting to correlate metabolic with physical change. Negative findings also need to be interpreted with caution and conclusions tempered accordingly when the statistical power of comparative analyzes is limited by insufficient sample size, an almost unavoidable consequence during investigation, for example, of "HAPE susceptibles".

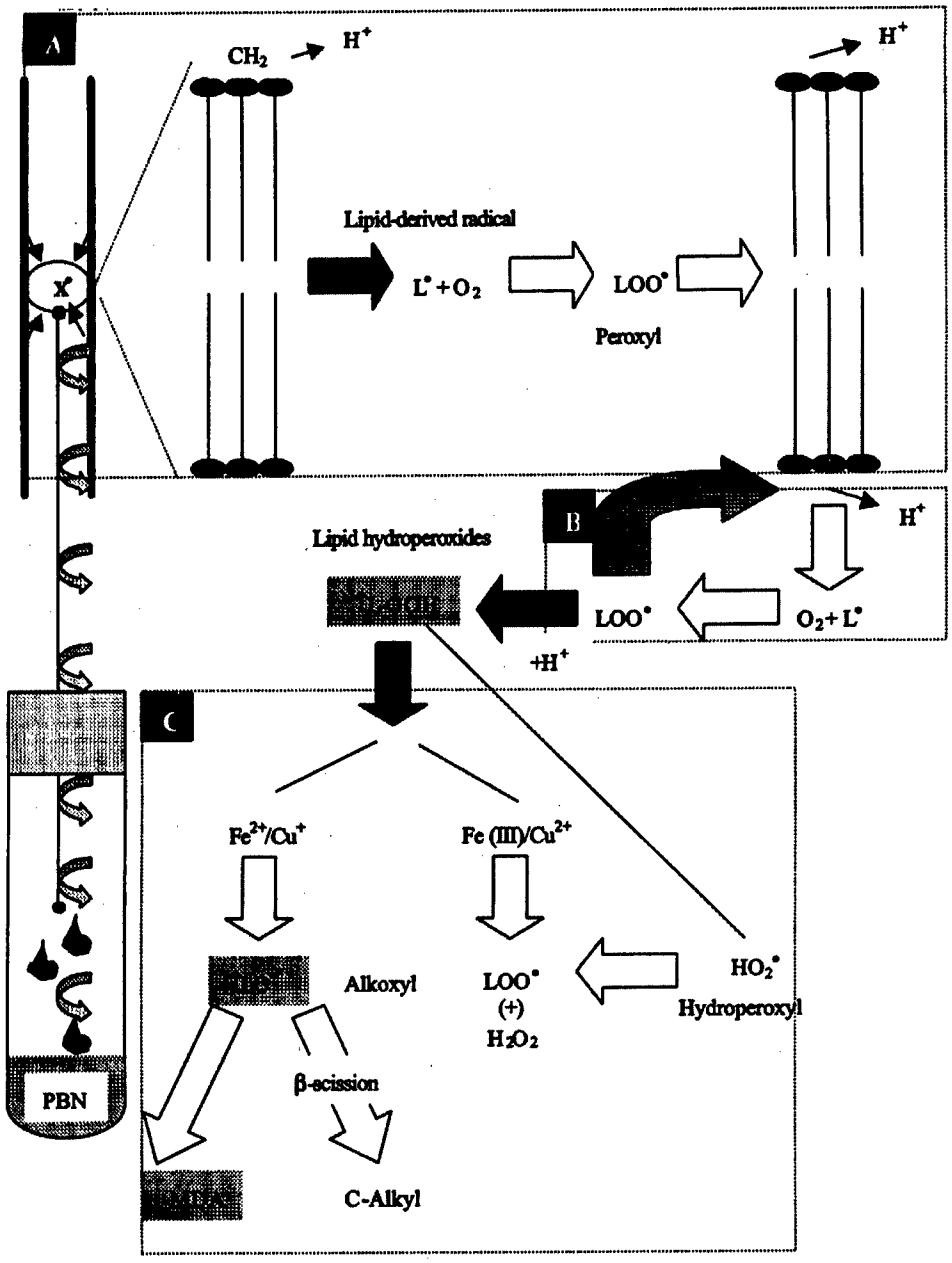


Figure 4. A schema of the proposed sequence of events leading to the "downstream" EPR spectroscopic detection of lipid-derived alkoxyl radicals. X• refers to "initiating" free radical species that may be O_2, C or N_2-centered, attacking PUFA-rich circulating lipids and/or cell membranes. Stippled boxes refer to reaction cascades (A = radical initiation, B = propagation, C = decomposition). Shaded intermediates indicate markers typically measured and corresponding changes at high-altitude.

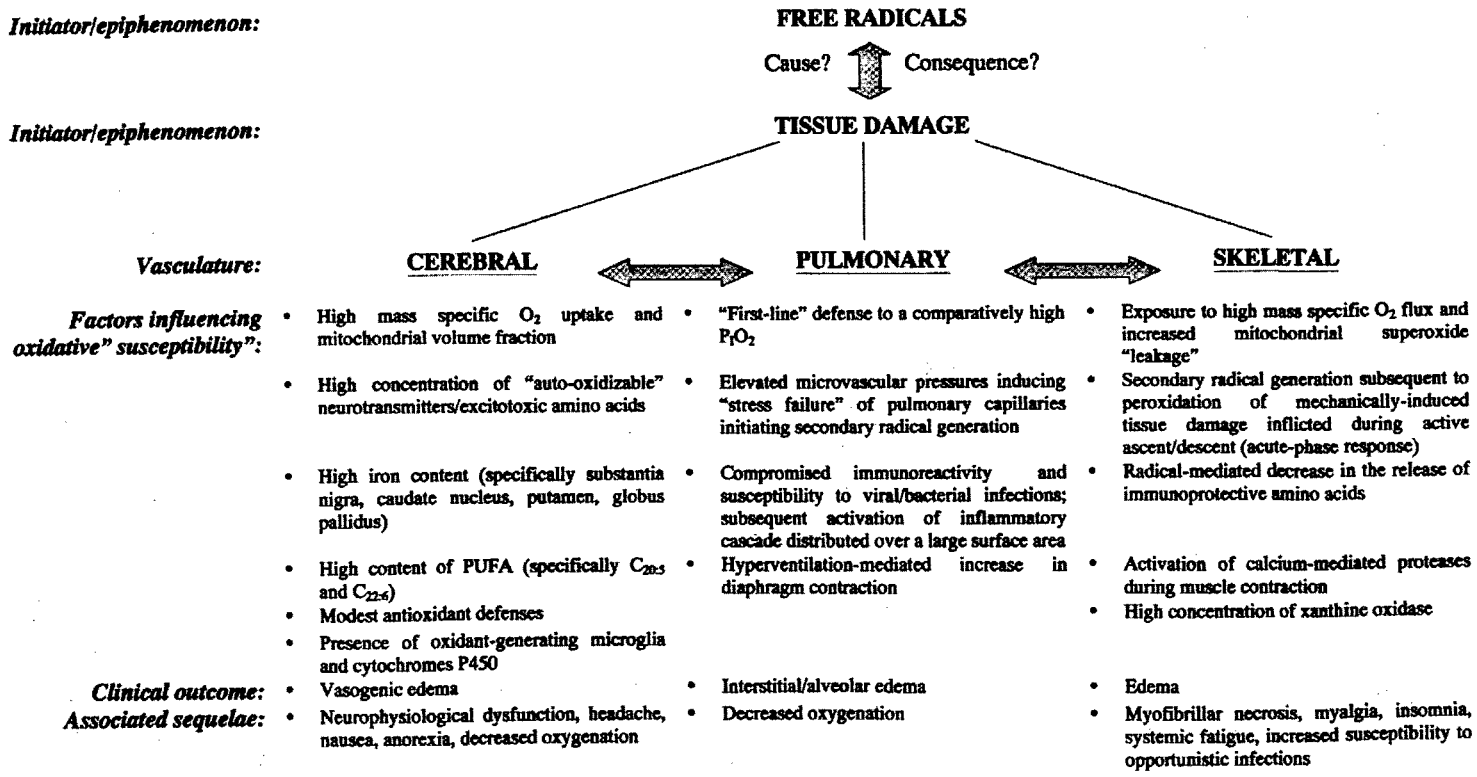


Figure 5. Vascular susceptibility to oxidative tissue damage and etiology of altitude illness; cause or consequence?

Though recent studies have indicated HAPE to be a non-inflammatory hydrostatically-mediated breach of the alveolar-capillary membrane (16, 25), the possibility of primary *radical-mediated* vascular damage still remains to be explored with any real conviction. This is especially true when considering the potential mechanisms associated with the more complex pathologies of AMS and HACE. Imaging studies combined with the simultaneous, direct assessment of radical formation and specific molecular markers of blood-brain-barrier damage in the cerebrospinal fluid of the "healthy and ill brain" are to be encouraged, in addition to catheter studies examining radical "exchange" across this elusive organ. *Entre-temps*, the data presented in Figure 7 derived from an uncharacteristically large sample, hint tentatively at the possibility of global and indiscriminate tissue damage that remains to be excluded as an initiating phenomenon. In contrast, preliminary findings investigating changes in localized markers of cerebral tissue damage such as neuron specific enolase were surprisingly unremarkable (Bailey and Bärtsch, unpublished observations).

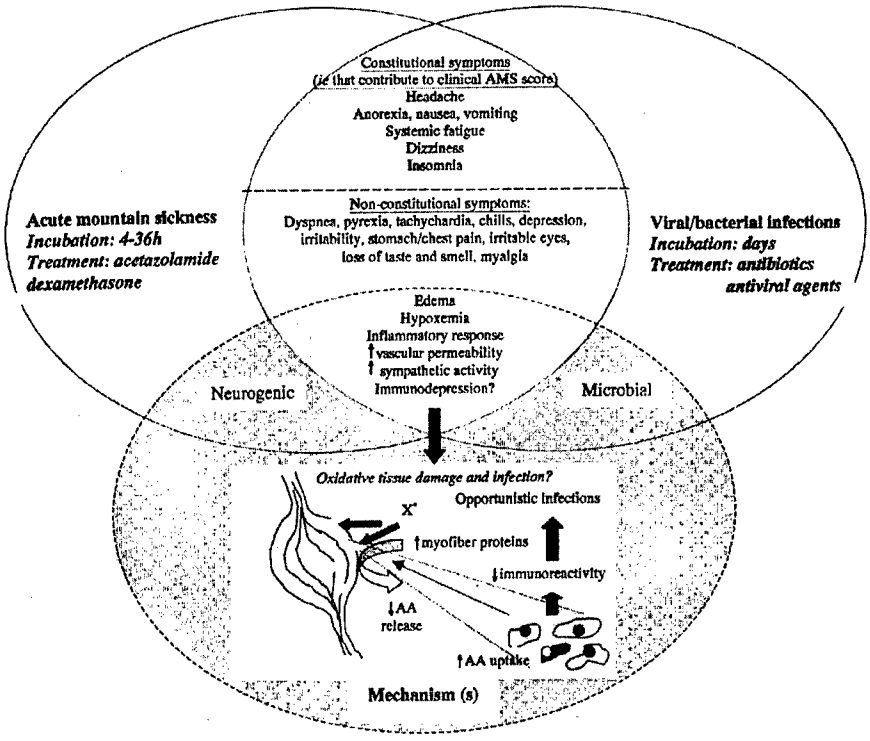


Figure 6. Shared and independent symptoms/pathophysiology of acute mountain sickness (AMS) and infection at high-altitude. Free radical-mediated damage to the transporter system in the muscle membrane may influence the supply of immunostimulatory amino acids (AA), in particular glutamine, since this is the flux-generating step for its release from skeletal muscle (18). Local demand may also increase to support the energetic requirements of activated immune cells during migration to injured tissue, further compounding substrate availability and thus increasing individual susceptibility to "opportunistic infections".

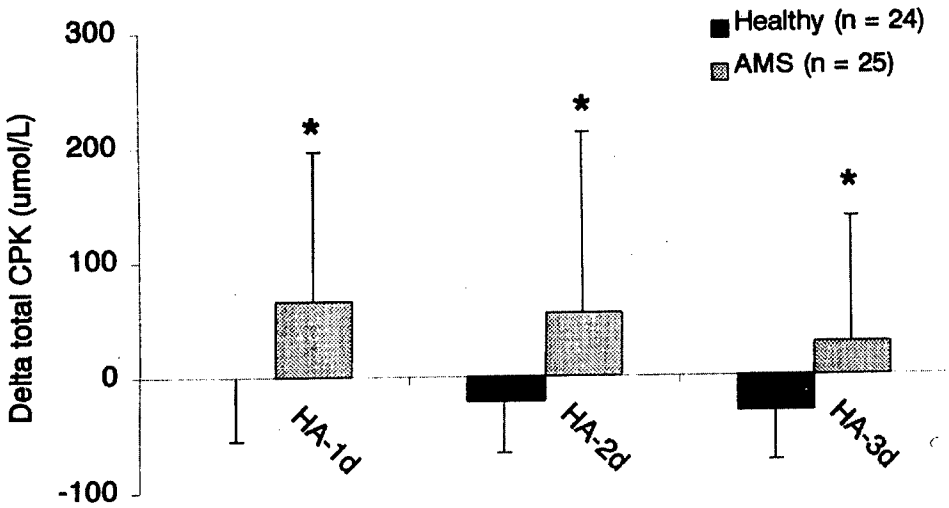


Figure 7. Temporal changes in total creatine phosphokinase (CPK) activity at 4,559m in subjects with and without AMS. Each histogram was calculated by subtracting the pre-ascent control value from each of the respective time points at high-altitude, expressed as the mean ± SD difference. Subgroups categorized as those who were free of any clinical symptoms of AMS or HAPE (Healthy) and those with AMS ($n = 15$) and HAPE ($n = 10$). *different between groups ($P < 0.05$).

Interventional Studies

Follow-up placebo-controlled, double-blind studies (3) have subsequently demonstrated a moderate improvement, though not complete reduction, in constitutional symptoms following prophylactic antioxidant vitamin supplementation (Figures 8). These data are the first to indicate that free radicals and associated tissue damage may contribute, at least in part, to the pathophysiology of altitude illness. A brief summary of the sequence of associated events that may contribute to the clinical symptoms of AMS and HACE is presented in Figure 9.

A RADICAL SOURCE; CELLULAR OXYGENATION *VS.* FLUX

What are the principal oxidant generators at high-altitude? Most scientists would concede to laboratory-based research to answer this challenging question, complicated by the numerous stressors that add to the "radical burden" in an environmental extreme. The final chapter of this review will briefly present preliminary evidence for an emerging concept that may facilitate our understanding of the "free radical paradox"; the interacting effects of exercise and inspiratory hypoxia are central to this tenet.

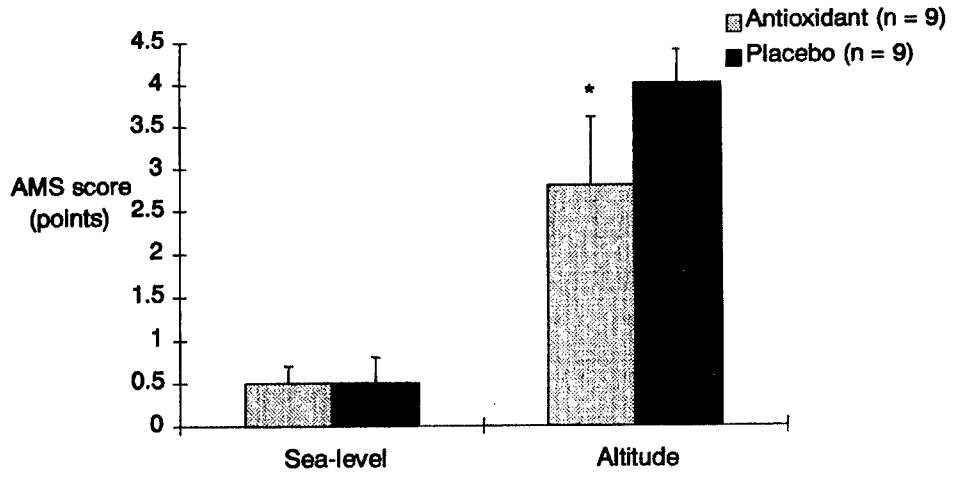


Figure 8. Prophylactic benefits of antioxidant vitamin supplementation against AMS during ascent to Mt. Everest basecamp at 5,180m: sea-level data points represent mean values obtained over a 7 day period; altitude data points represent mean of morning and evening measurements during a 10 day ascent; main effects for location (sea-level *vs.* altitude, $P < 0.05$) and group (antioxidant *vs.* placebo, $P < 0.05$) and interaction effect for group *x* location ($P < 0.05$). * difference between groups as a function of location ($P < 0.05$).

Exercise and Cellular Oxygenation

For almost half a century, physiologists have suggested that acute exercise results in increased free radical generation and the associated implications, both in health and disease, has proven the subject of much intrigue and speculation. Mitochondria are considered the principal "radical reactors" and it has been estimated that between 1-2% of total electron flux can undergo univalent reduction at the NADH dehydrogenase (26) and/or ubiquinone cytochrome bc segment of complex III (20) in the mitochondria to form the superoxide anion, the stoichiometric precursor to hydrogen peroxide. Not-withstanding other potential sources, a mass action effect initiated by a systemic increase in oxygen uptake ($\dot{V}O_2$) has been implicated as the primary mechanism responsible for exercise-induced free radical generation (24). However, a combined reliance on indirect and therefore potentially circumstantial biomarkers confined to the venous circulation and exercise models that typically recruit heterogenous muscle groups confounded by a substantial isometric component may have seriously influenced prior interpretation of the source and mechanisms associated with exercise-induced free radical generation. In brief, there is still no evidence that clearly demonstrates that contracting human skeletal muscle actually generates free radicals in spite of widespread speculation! Furthermore, our findings have consistently demonstrated evidence for comparatively greater maximal exercise-induced free radical generation in both acute and chronic hypoxia despite markedly lower $\dot{V}O_2$'s; findings that clearly challenge the "flux concept" (2, 4).

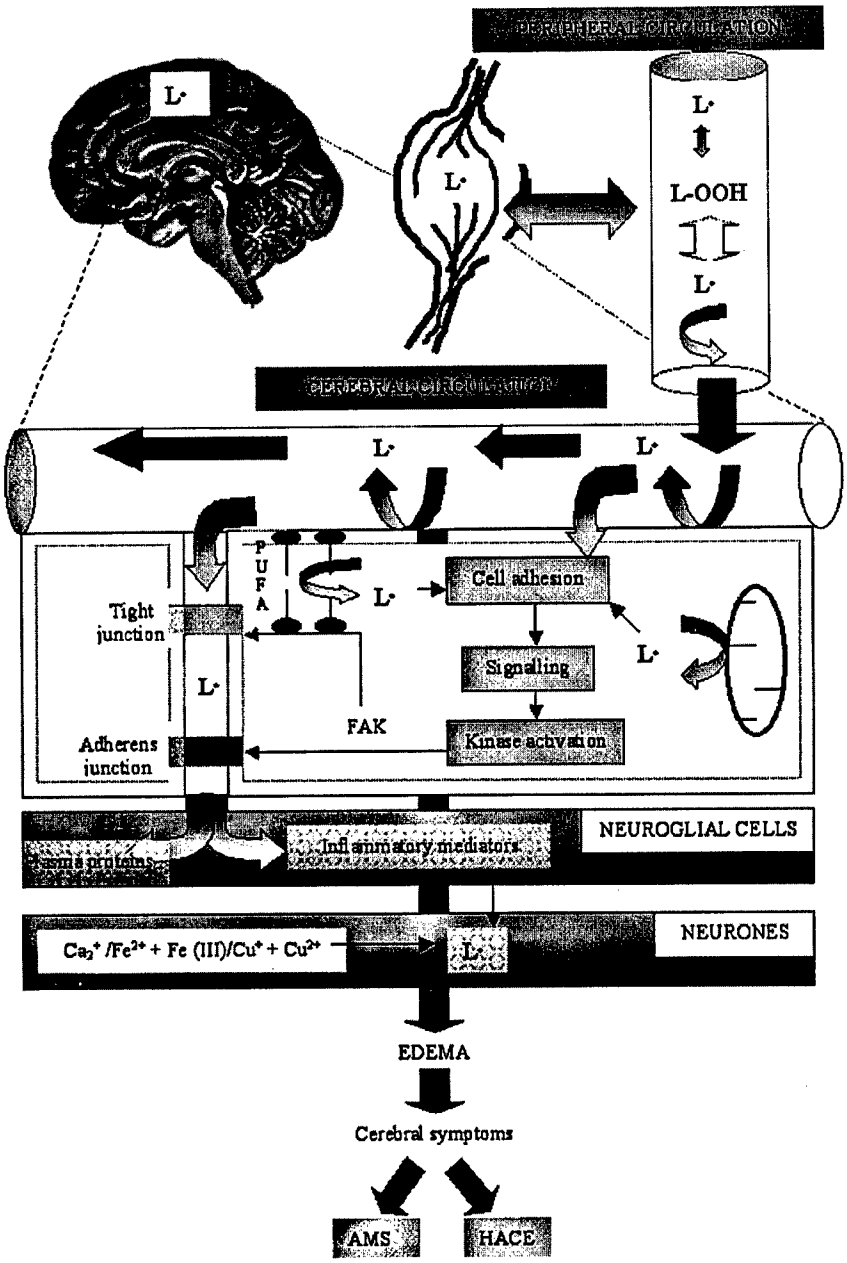


Figure 9. Hypothetical model outlining radical-mediated contributions to AMS and HACE. Cascading lipid-derived radicals detected in the peripheral circulation "feed" into the cerebral circulation, activating a sequence of events in the endothelia. These may include phosphorylation of intercellular adhesion molecules (*eg.* ICAM-1) that could activate kinases (*eg.* FAK, focal adhesion kinase) and induce cytoskeletal damage and thus compromise junction integrity. Increased paracellular permeability could lead to subsequent "leakage" of mediators and oxidant propagation activating adjacent cells to promote formation of edema.

Methodological Advances

To resolve these uncertainties and extend our preliminary findings, we decided to combine EPR spectroscopy with *ex-vivo* spin trapping and data obtained using ^{1}H magnetic resonance spectroscopy for the direct assessment of free radicals and intracellular PO_2 (iPO_2) respectively (6, 8). Single-leg knee extensor (KE) exercise was specifically chosen as the exercise paradigm because it affords the unique opportunity to examine contracting skeletal muscle and associated vasculature in a functionally isolated scenario. Using these direct techniques and the sampling of arterial/venous blood combined with the simultaneous measurement of femoral venous blood flow ($\dot{Q}$), we hypothesized that incremental physical exercise in normoxia would result in a net free radical outflow from the quadriceps femoris muscle bed. We further hypothesized that a decrease in intracellular oxygenation (as assessed via iPO_2) would compound the anticipated increase in outflow in response to an exercise-induced increase in O_2 flux to respiring tissue.

The EPR spectra presented in Figure 10 provides the first evidence of simultaneous PBN spin adduct detection in the femoral arterial and femoral venous circulation of a subject performing KE exercise. Visual inspection of these spectra and quantitative findings illustrated in Figure 11I clearly indicate a veno-arterial spin adduct concentration difference ($v\text{-}a_{diff}$) across the active muscle bed. When combined with the observed rise in $\dot{Q}$ this resulted in net adduct outflow (Figure 11II). An increase in the $v\text{-}a_{diff}$ and thus outflow was only apparent between the low (24 ± 6 WR_{MAX}) to moderate ($66 \pm 5\%$) intensity domains and not between the moderate to high ($98 \pm 4\%$) domain despite further increases in leg $\dot{V}O_2$.

A detailed examination of the individual components of convective O_2 transport identified that the primary factor associated with outflow was $\dot{Q}$ and not the peripheral extraction of O_2 by muscle (Figure 12II), which remained essentially invariant with increasing exercise intensity. Blood flow is a well established physiological stimulus for vascular endothelial O_2 and N_2-centered free radical release (14) and may have contributed to the signals observed in the present study.

However, when adduct release was expressed relative to $\dot{Q}$ it became clear that an alternative mechanism was potentially operant. Figure 13 demonstrates that (normalized) release responded with remarkable precision to existing data for exercise-induced changes in iPO_2. Richardson *et al.* (21, 22) have consistently demonstrated a marked decrease in iPO_2 between the low to moderate intensity domains specifically employed in the present study whereas no changes have been observed between the moderate to high domains, a consequence of increased muscle O_2 diffusional conductance. These findings provide the first direct and quantitative evidence for an increase in free radical outflow from an active skeletal muscle bed in humans that preliminary indications suggest is PO_2 (and not purely flux)-dependent.

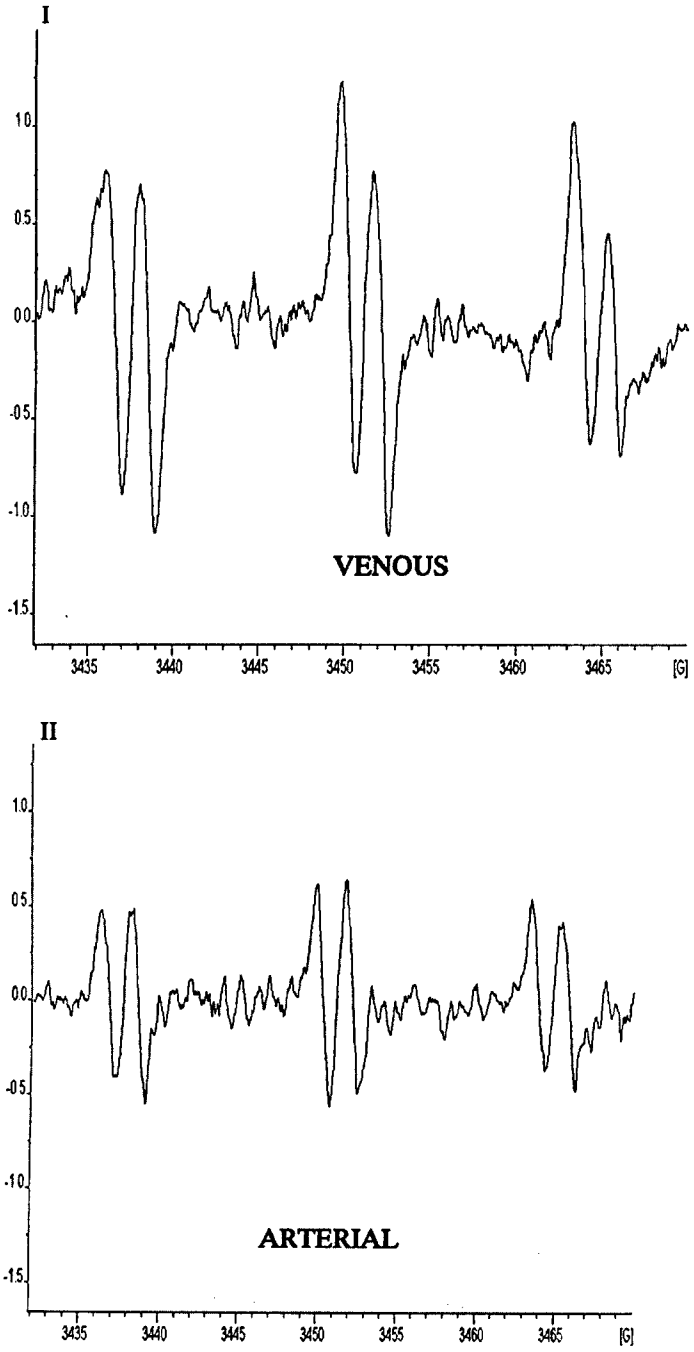
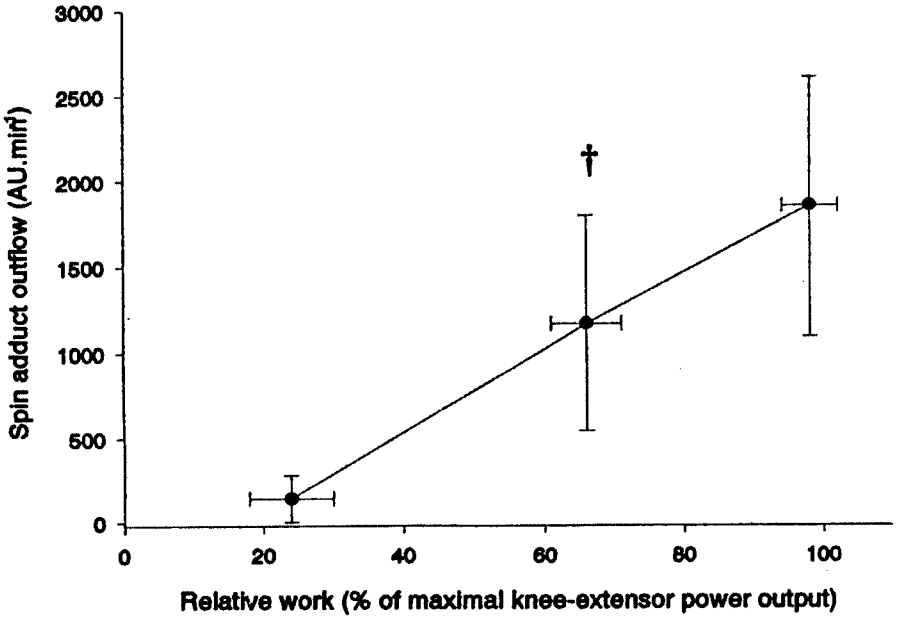


Figure 10. Typical EPR spectral signals of a PBN spin adduct detected in the femoral arterial (I) and venous (II) circulation at 70% WR_{MAX}. Note the comparatively greater signal intensity for the venous sample.



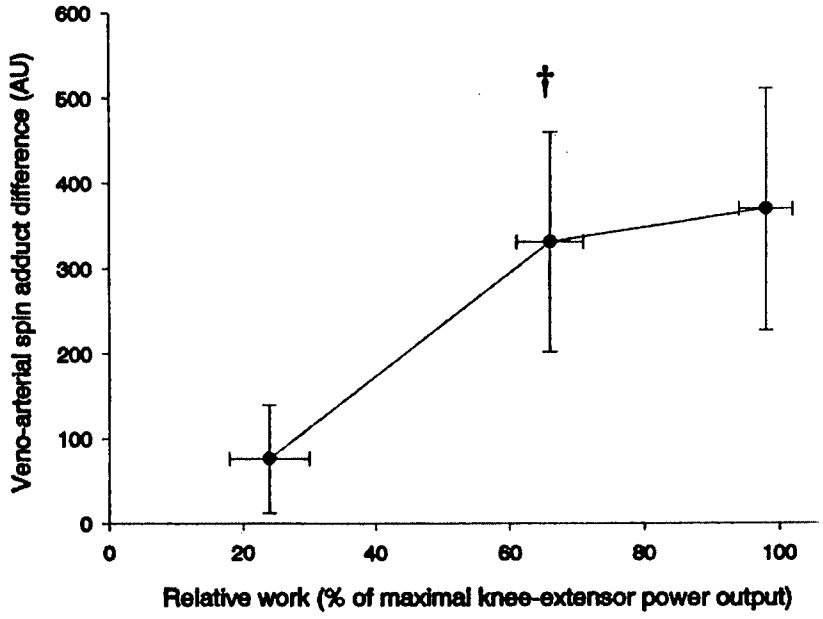


Figure 11. Effects of relative work intensity on PBN spin adduct veno-arterial concentration difference (I) and net spin adduct outflow (II). Net outflow was calculated as the product of the veno-arterial concentration difference and femoral venous blood flow. † different compared to preceding value ($P < 0.05$).

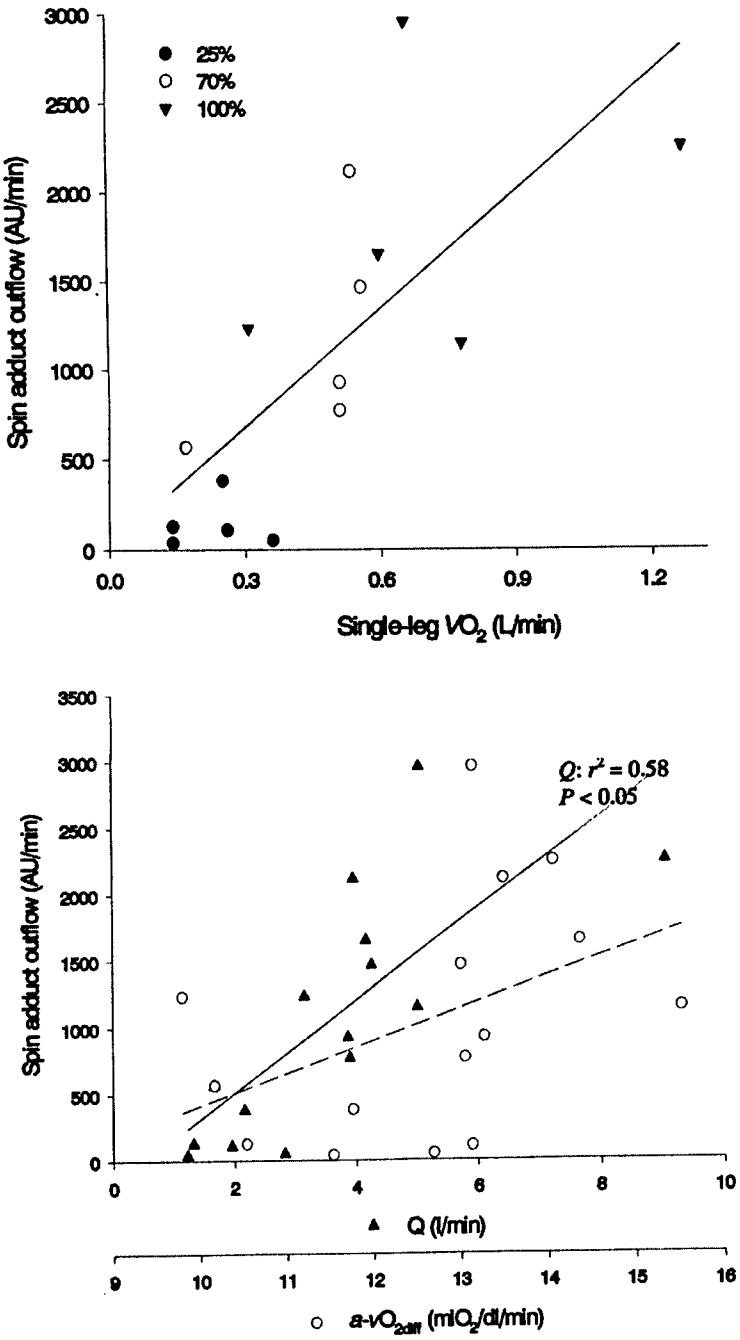


Figure 12. Relationship between (I) spin adduct outflow and single-leg oxygen uptake ($\dot{V}O_2$) and (II) individual components of convective O_2 transport. Femoral venous blood flow ($\dot{Q}$) was clearly the contributory factor responsible for the relationship between outflow and $\dot{V}O_2$.

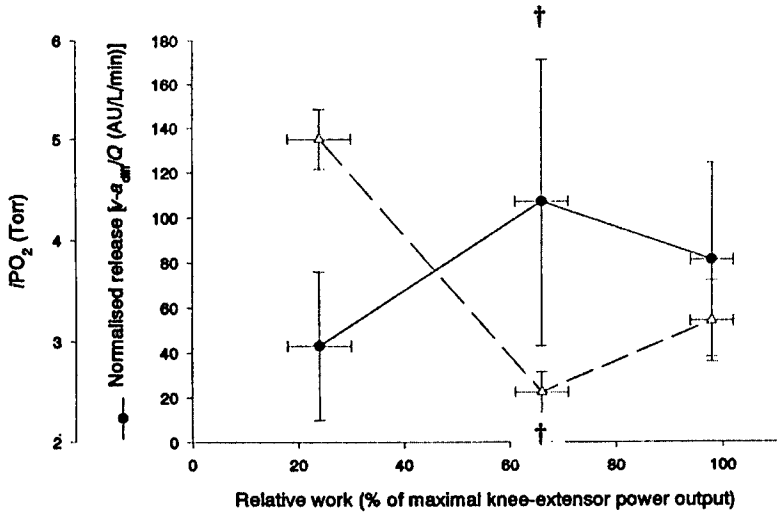


Figure 13. Relationship between intracellular PO_2 (iPO_2) and spin adduct release normalised for femoral venous blood flow during incremental exercise. † different compared to preceding value ($P < 0.05$).

FUNCTIONAL SIGNIFICANCE AND CLOSING REMARKS

Figure 14 represents a hypothetical model based on recent *in-vitro* findings (11) to explain the functional significance and potential mechanisms associated with PO_2 (as opposed to purely O_2 flux)-dependent free radical release by respiring tissue. An exercise or altitude-induced decrease in iPO_2 may induce a decrease in the V_{MAX} of cytochrome oxidase and thus increase the reduction state of mitochondrial electron carriers upstream of cytochrome aa_3. An increased mitochondrial redox for any given O_2 (hence e^-) flux could theoretically compound mitochondrial $\dot{O}_2^-$ generation by increasing the lifetime of reduced electron carriers, specifically ubisemiquinone. The traditional notion that exercise-induced free radical generation is a maladaptive phenomenon due to the indiscriminate damage to organic molecules therefore needs to be reconsidered. These pleitropic biomolecules may prove important "second-messengers" (14), key elements in an elaborate signal transduction cascade initiated by the mitochondrion as the primary O_2 sensor, that can respond to subtle alterations in intracellular oxygenation, adjust homeostasis and initiate protective adaptation. Future understanding of what constitutes physiologically useful *vs.* physiologically excessive oxidant generation at high-altitude may help define the fine line that dictates a mountaineer's health or illness, summit success or failure.

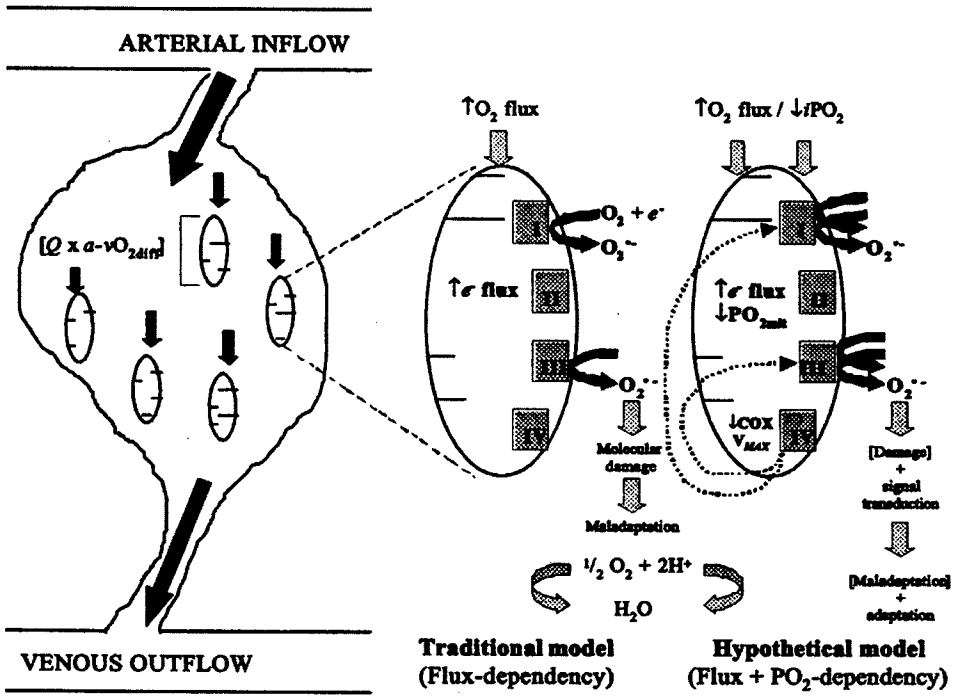


Figure 14. Pictorial representation of exercise-induced mitochondrial free radical generation. In the traditional model, mitochondrial electron "leakage" during the tetravalent reduction of molecular O_2 to H_2O and subsequent generation of free radicals (predominantly at complexes I and III of the METC) can initiate and propogate cellular membrane damage; largely a maladaptive phenomenon dependent on $\uparrow e^-$ flux. In the revised, PO_2-dependent model, the mitochondrion is considered the primary O_2 sensor that can respond to subtle alterations in intracellular O_2 homeostasis by initiating an elaborate signal transduction cascade to invoke adaptive responses to the stressor. A decrease in intracellular PO_2 (and potentially mitochondrial PO_2) may decrease cytochrome oxidase (COX) V_{MAX}, causing an accumulation of electrons in the reduced state thus increasing the mitochondrial generation of free radicals. In this sense, free radicals can be considered as putative signal transductants, still capable of causing molecular damage, but only when in physiological excess.

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Chapter 16

HYPOXIC REGULATION OF BLOOD FLOW IN HUMANS

Skeletal muscle circulation and the role of epinephrine

John R. Halliwill

Abstract: Vascular tone represents the balance between local vasodilator mechanisms which attempt to secure adequate blood flow for metabolic demand and neural vasoconstrictor reflexes attempting to maintain arterial pressure. Hypoxia alters vascular tone, shifting this balance in complex ways. Hypoxic vascular responses are not uniform across vascular beds and the mechanisms of hypoxic vasodilation appear to be tissue specific. In healthy humans, skeletal muscle vascular beds exhibit a graded vasodilation in response to hypoxia despite increases in sympathetic vasoconstrictor nerve activity. Previous studies have documented a number of vasodilator substances or systems that appear to be involved in this hypoxic vasodilation. My colleagues and I have conducted studies on the extent to which sympathetic vasoconstriction can mask hypoxic vasodilation, and how sympathetic vasoconstrictor activity interacts with local factors that mediate hypoxic vasodilation in humans. We have focused largely on β-adrenergic mediated vasodilation, noting that it produces some of its effects via a nitric oxide (NO) pathway. This review will explore the role of epinephrine in generating skeletal muscle vasodilation. How the many factors that determine vascular tone during hypoxic stress impact on the regulation of arterial pressure and how hypoxic vasodilation is altered in several pathophysiological conditions will be discussed.

Key Words: altitude, sympathetic nervous system, syncope, vasodilation, orthostasis

INTRODUCTION

Hypoxia can have profound influences on the circulation. Whether net vasoconstriction or vasodilation occurs in a vascular bed is dependent upon balance between the local

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

effects of hypoxia and changes in neural control of vascular tone produced by systemic hypoxia (19, 26). Considerable evidence demonstrates that this balance is dependent on species, vascular region, and the degree of hypoxia. Striking differences have been observed between the responses of human vascular beds and animal vascular beds to hypoxia produced by spontaneous breathing of hypoxic gas mixtures (9, 43); thus, the primary focus in this review and it's companion reviews (13, 27) will be on the known vascular responses in humans. This review will focus on the balance between vasoconstrictor and vasodilator signals in the skeletal muscle circulation, highlighting the role of epinephrine as an important and complicated vasodilator signal.

VASODILATOR-VASOCONSTRICTOR BALANCE

Vascular tone represents the balance between local vasodilator mechanisms which attempt to secure adequate blood flow for metabolic demand and neural vasoconstrictor reflexes attempting to maintain arterial pressure. The vascular smooth muscle is continuously exposed to varying concentrations of vasoactive substances, including endothelium-derived factors such as NO and EDHF, prostaglandins, adenosine, ATP, and K^+ and H^+ ions. Furthermore, circulating levels of epinephrine, blood gas levels, and osmolarity can affect the vascular smooth muscle contractile state. Figure 1 depicts these various factors involved in vasodilator-vasoconstrictor balance.

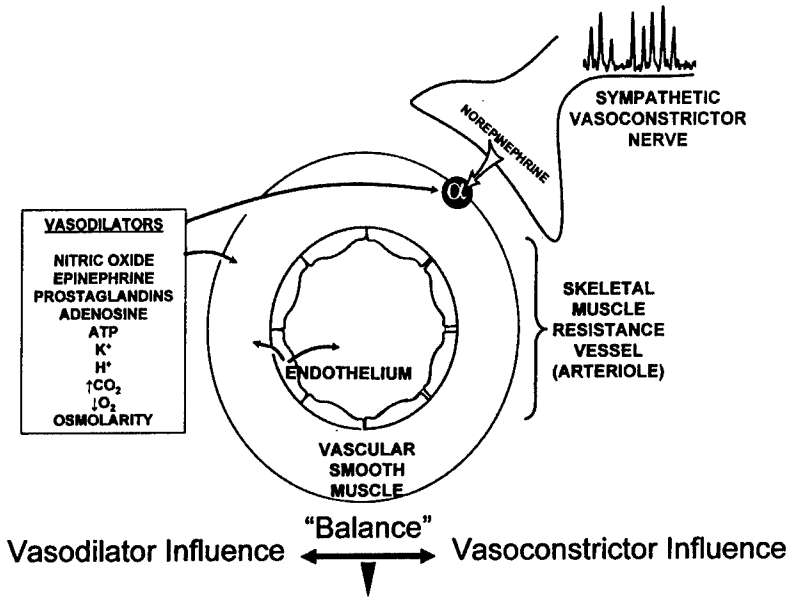

Figure 1. Vasodilator-vasoconstrictor balance. Numerous substances that are released locally from tissue, smooth muscle, and the endothelium contribute to vasodilator tone. These signals are balanced by vasoconstrictor tone, largely the product of tonic sympathetic vasoconstrictor nerve activity and the release of norepinephrine. The balance of these two influences determines vascular tone.

Hypoxia alters the balance of vascular tone, but responses are not uniform across vascular beds and the mechanisms of hypoxic vasodilation appear to be tissue specific as indicated in Figure 2. In humans, during moderate hypoxia, the cerebral and coronary vascular beds vasodilate (22, 41) and a modest renal vasodilation is sometimes seen (2, 3, 8). The splanchnic bed shows a graded dilation in response to moderate and severe hypoxia in humans (35). Likewise, whole-limb vascular beds show a graded dilation in response to moderate and severe hypoxia in humans (20, 39). Whole-limb vascular responses reflect changes in both skeletal muscle vascular beds and cutaneous vascular beds, with conventional wisdom suggesting that the dilator response to hypoxia largely occurs in the skeletal muscle vascular beds. In contrast, vasoconstriction is seen in the hand during moderate and severe hypoxia. Since hand blood flow reflects a greater proportion of flow to skin than muscle, this has further contributed to the widely held belief that in the limbs of humans, only skeletal muscle vascular beds and not the cutaneous vascular beds participate in hypoxic vasodilation (20, 39). However, this concept is currently being contested (27).

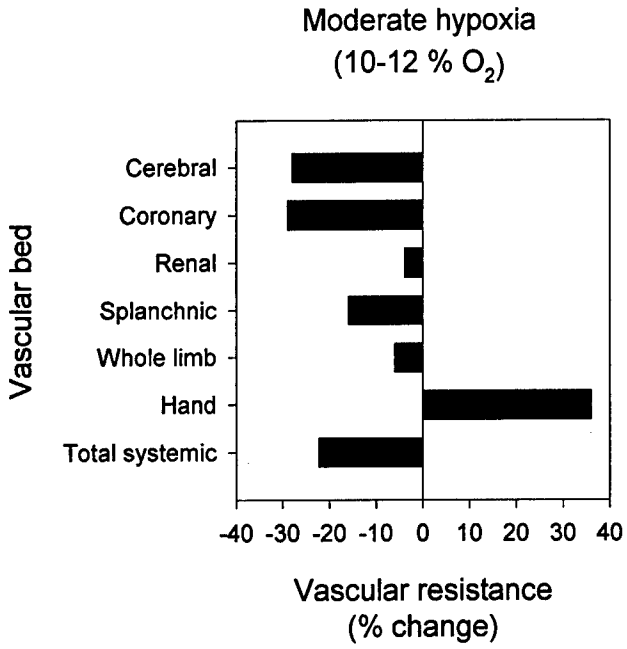

Figure 2. Vascular responses to moderate hypoxia differ across peripheral vascular beds. Depicted responses represent values reported from numerous sources (2, 3, 8, 20, 22, 27, 35, 39, 41).

Figure 3 presents some basic observations on the nature of vasodilation in the human forearm, which has been extensively used as a model for investigating skeletal muscle responses. Notably, vasodilation is graded to the degree of hypoxia, in terms of inspired O_2 level or arterial O_2 saturation level. From an experimental design perspective, it is convenient that responses are equal bilaterally and highly reproducible during multiple brief

exposures. This has facilitated the study of hypoxic responses in skeletal muscle vascular beds, as specific agonists and antagonists can be selectively infused into one arm, leaving the contralateral arm to serve as an experimental control.

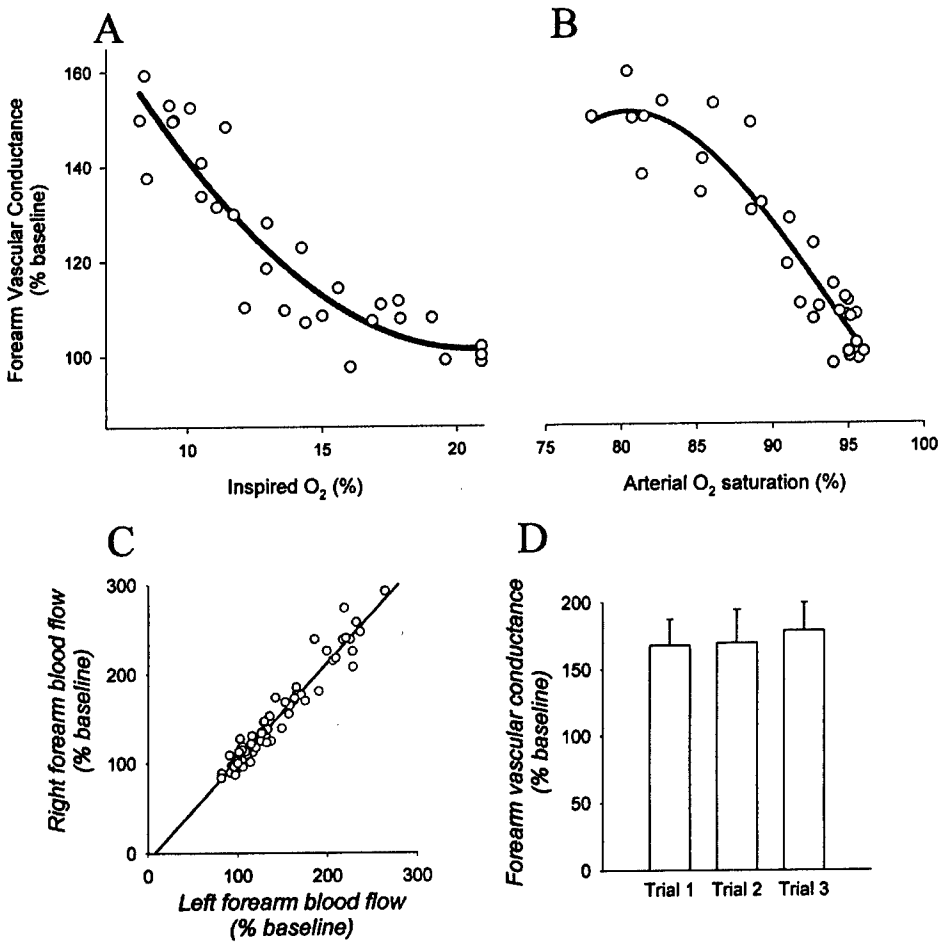

Figure 3. Forearm vasodilator responses to hypoxia. A and B: Forearm vasodilation is graded to the degree of hypoxia, in terms of inspired O_2 level or arterial O_2 saturation level. C: Forearm vasodilation is equal bilaterally. D: Forearm vasodilation is reproducible during multiple brief exposures to hypoxia. (Halliwill, unpublished observations).

It must be emphasized that these overt vasodilator effects may overlie an unknown degree by sympathetic vasoconstriction. (However, sympathetic vasoconstriction itself may be attenuated by hypoxia, a topic reviewed in the next chapter (13).) In other words, frank vasodilation is observed in human limbs despite, or in the face of, a profound rise in

sympathetic vasoconstrictor nerve activity. Thus, the degree of vasodilation substantially "outweighs" the vasoconstrictor response in determining vasomotor tone during hypoxia.

SYMPATHETIC VASOCONSTRICTION IN SKELETAL MUSCLE

Hypoxia leads to considerable reflex autonomic changes in respiratory and cardiovascular system function. Acute hypoxia increases sympathetic vasoconstrictor outflow to muscle vascular beds (36, 40) by resetting baroreflex control of muscle sympathetic nerve activity to higher pressures and higher levels of sympathetic nerve activity, without changes in sensitivity of the arterial baroreflex (18;Figure 4). Interestingly, sympathetic activation during hypoxia occurs without measurable increases in plasma norepinephrine, which suggests that hypoxia may produce either a decrease in release of norepinephrine or an increase in the reuptake of norepinephrine by sympathetic nerves. Leuenberger *et al.* have documented increased norepinephrine clearance during acute hypoxia (23), which may be part of the explanation.

Weisbrod *et al.* (44) asked the question, how much greater would hypoxic vasodilation be without this rise in sympathetic vasoconstrictor nerve activity? Using selective regional α-adrenergic receptor blockade in the forearm, they demonstrated that activation of sympathetic vasoconstrictor nerves masks to a substantial degree the effects of hypoxia on vascular tone in the skeletal muscle vascular beds of the human forearm. As Figure 5 shows, hypoxic vasodilation is substantially larger in the "experimental" arm, which had received phentolamine (an α-adrenergic blocker), than in the control arm (no blockade). By removing the competing influence of changes in sympathetic nerve activity, this experimental paradigm also documents that the sympathetic nerves can mediate vasoconstriction under hypoxic conditions. What are the implications for superimposed sympathetic vasoconstriction and hypoxic vasodilation? It may be that hypoxic vasodilation helps to maintain adequate blood flow on the local level, and that the sympathetic vasoconstrictor response represents a "safety mechanism" preventing widespread activation of vasodilation from outstripping cardiovascular reserves. Indeed, excessive hypoxic vasodilation could result in the orthostatic intolerance or hypotension that has been reported in some individuals during acute systemic hypoxia (1, 21, 34)(see below).

Regardless of the physiological implications, superimposed sympathetic vasoconstriction represents a major experimental confound when attempting to study the mechanisms of vasodilation in humans. One could argue that in order to determine the contribution of a particular vasodilator to the overall hypoxic response, the system is best studied under conditions of α-adrenergic blockade.

SKELETAL MUSCLE VASODILATION: THE USUAL SUSPECTS

Previous studies have documented a number of vasodilator substances or systems that appear to be involved in hypoxic vasodilation. In humans, the skeletal muscle vasodilation seen during severe hypoxia can be reduced by β–adrenergic blockade (4, 33, 44), suggesting it is mediated by a β–adrenergic pathway (e.g., Figure 5, trial 2). Accordingly, hypoxia

increases circulating levels of epinephrine approximately two-fold under these conditions (44). It is unclear whether this is due to an increase in sympathetic nerve activity to the adrenal gland or due to a direct effect of hypoxia on the adrenal gland. Nonetheless, these findings indicate that epinephrine plays a substantial role in mediating the hypoxic vasodilation, overriding the effects of sympathetic vasoconstriction in healthy humans. In the study shown in Figure 5, observations were done in the presence of α-adrenergic receptor blockade, extending prior observations by quantifying how much vasodilation can be attributed to this mechanism. Roughly 50 % of the total vasodilator response to an arterial O_2 saturation of 85 % can be attributed to activation of β-adrenergic receptors in skeletal muscle vascular beds.

However, a study by Blitzer *et al.* (5) suggests that hypoxic vasodilation in skeletal muscle is mediated by NO. They found that NO synthase inhibition blocked ~ 55 % of the hypoxic vasodilator response. It should be noted that studies have demonstrated that the skeletal muscle vasodilation which occurs during several sympathoexcitatory maneuvers is often dependent upon an interaction between NO and β–adrenergically mediated vasodilation (12, 17, 31). A plausible scenario is that NO functions in part as a final pathway for vasodilation that is secondary to activation of β–adrenergic receptors by circulating epinephrine. In fact, Dawes *et al.* (11) found that 50-60 % of the dilation produced by intra-arterial infusion of β–adrenergic agonists in humans is dependent on NO (blocked by L-NMMA). Thus, in the study by Weisbrod *et al.* (44), the NO synthase inhibition performed by Blitzer *et al.* (5) was repeated subsequent to β–adrenergic receptor blockade. By comparing trials 2 and 3 in Figure 5, it can be seen that without functional β-adrenergic receptor, NO synthase inhibition had no effect on hypoxic vasodilation. Taken together, the data from Blitzer *et al.* and Weisbrod *et al.* are consistent with hypoxia eliciting NO-dependent vasodilation exclusively via stimulation of β–adrenergic receptors.

Since considerable dilation persists in the presence of both β -adrenergic blockade and NO synthase inhibition, it is likely that an additional vasodilator mechanism is activated by hypoxia in the skeletal muscle vascular beds of humans. Further, animal studies and studies in isolated vessels indicate that ATP-sensitive potassium channels have an extensive role in mediating hypoxic vasodilation (10, 42) and that these channels are activated by adenosine released from the endothelium (6, 7). Adenosine levels in skeletal muscle are increased in humans during hypoxia (25), and Leuenberger *et al.* (24) found that aminophylline, an adenosine-receptor antagonist, can reduce the hypoxic vasodilator response by approximately 80 % in humans. It is important to recognize that this study (as well as the study by Blitzer *et al.* mentioned above) did not quantify vasodilator responses in the presence of α-adrenergic blockade and that it was under these conditions that aminophylline blocked 80 % of the apparent hypoxic vasodilation. In other words, the contribution of adenosine was likely to have been overestimated due to the effects of sympathetic vasoconstrictor activity (i.e., the apparent dilation may have represented only 40 % of the entire hypoxic vasodilation).

It would appear from this discussion that hypoxic vasodilation in skeletal muscle vascular beds of humans is due to both circulating epinephrine and locally produced adenosine, but that the relative contributions of these two vasodilator signals have yet to be defined. Furthermore, it is possible that additional substances are involved. The degree to which activation of sympathetic vasoconstriction during hypoxia can mask dilator responses and obscure study results has been largely unappreciated.

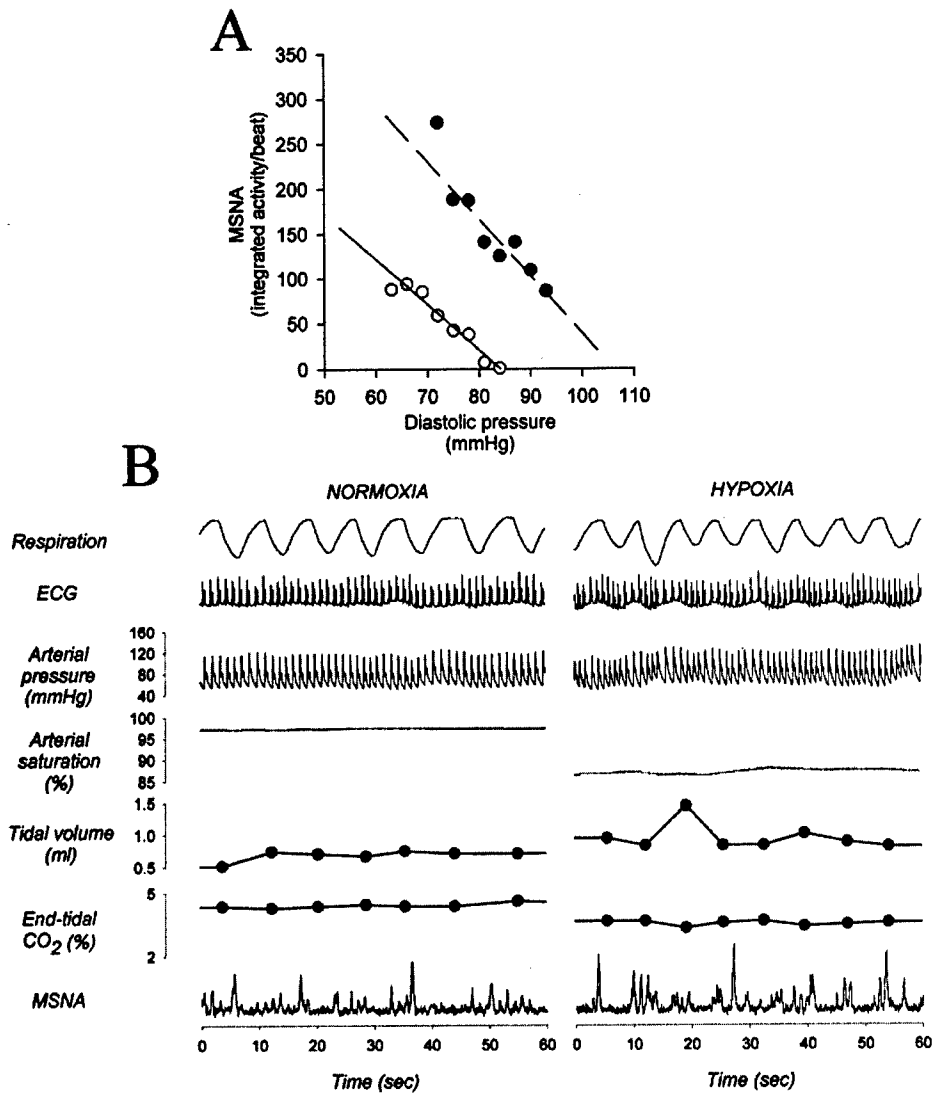

Figure 4. Hypoxia modifies the arterial baroreflex and increases sympathetic vasoconstrictor nerve activity. A: Data from a representative subject showing baroreflex relationship between muscle sympathetic nerve activity and diastolic pressure under normoxic conditions (open circles) and hypoxic conditions (filled circles). B: Representative tracing from a subject showing increased muscle sympathetic nerve activity (MSNA) during hypoxia compared to normoxia. Hypoxia resets the baroreflex to higher pressures resulting in elevated heart rates and increased sympathetic nerve activity. Modified from (18).

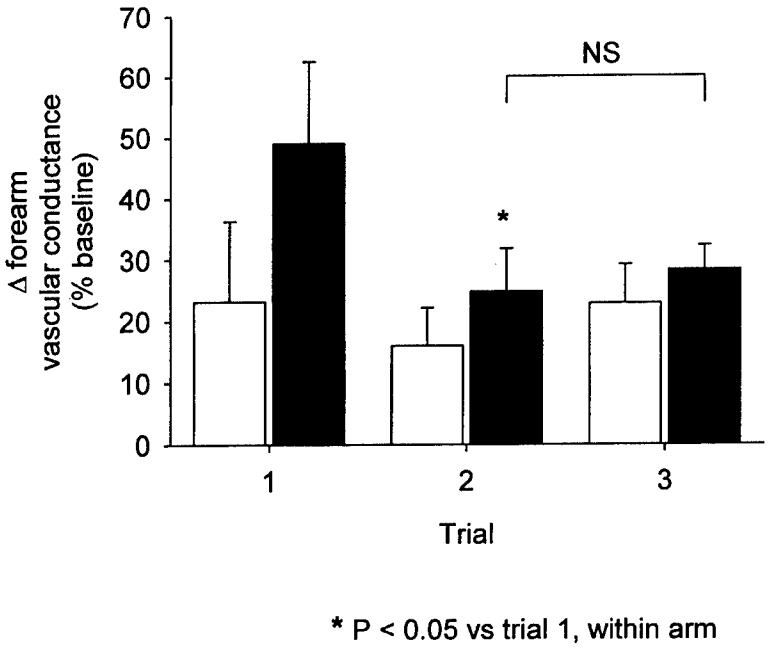

Figure 5. Forearm vascular conductance responses to hypoxia. The percent change in forearm vascular conductance in the control arm (open bars) and experimental arm (filled bars) during three trials of hypoxia. During all three trials, α-adrenergic receptors in the experimental arm were blocked with phentolamine, which augmented the hypoxic vasodilation. In trials 2 and 3, β-adrenergic receptors in the experimental arm were also blocked (propranolol), which blunted the hypoxic vasodilation. In trial 3, NO synthase was inhibited in the experimental arm, but this had no effect on hypoxic vasodilation. Modified from (44).

COMPLICATED VASODILATOR PATHWAYS

The role of NO in vasodilator responses is often complicated, and merits further mention. NO is often implicated in vasodilator responses based on the effect of NO synthase inhibition. However, this experimental approach provides limited information in that well-established second messenger models have demonstrated that often only basal levels of NO production are necessary for the expression of "normal" vasodilator responses. In other words, NO may play a permissive role in the activation of vasodilation by its mere presence at basal levels, and does not need to be activated as part of the response. Thus, vasodilation may be NO-dependent without necessarily being NO-mediated. In addition, the activation of NO production, when it occurs, is often a secondary pathway leading to vasodilation. This appears to be the case with activation of β–adrenergic receptors by circulating epinephrine during hypoxia. However, even in the case of β–adrenergically mediated vasodilation, it is still unknown whether the response to epinephrine is NO-dependent (NO merely exerts a permissive effect) or NO-mediated.

To further confuse these issues, recent work from Janice Marshall's lab (30) in animal models suggests that adenosine released from the endothelium during hypoxia interacts with NO via both a prostaglandin pathway and via activation of the ATP-sensitive K^+-channel. It is unclear at this time to what extent the role of adenosine in hypoxic vasodilation in humans can be explained by these same mechanisms.

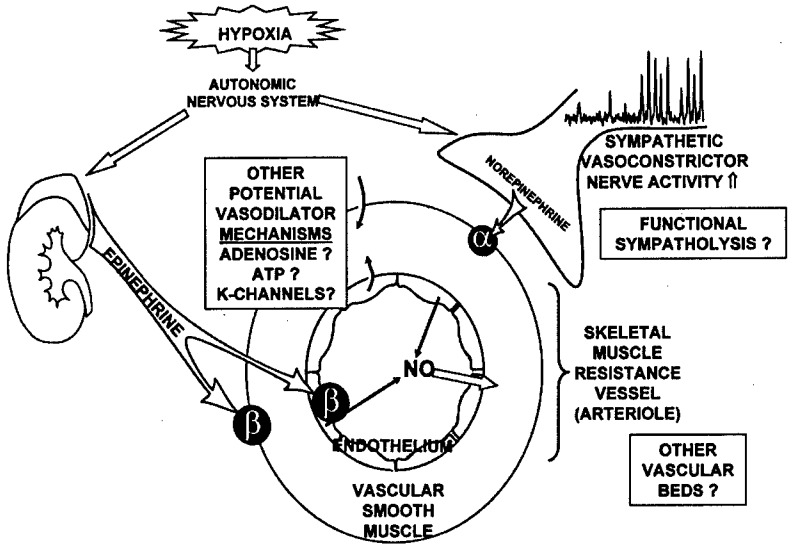

Figure 6. The "working model" for hypoxic vasodilation in skeletal muscle vascular beds in humans. A clear role has been demonstrated for β-adrenergic and α-adrenergic receptors. Strong evidence also supports a role for adenosine or a related compound, perhaps functioning through ATP-sensitive K^+-channels. It is also thought that NO from the endothelium may serve as a necessary component or final pathway for some of these substances. Finally, current studies are investigating the possibility that α-adrenergic pathways are modified during hypoxia (i.e., functional sympatholysis) and the extent to which this model is relevant to other vascular beds in humans (e.g., cutaneous vasculature).

One upcoming area of research in genetic polymorphisms may shed light on the inter-individual variation in hypoxic vasodilator responses in humans. A common polymorphism in the β_2-adrenergic receptor has been identified (substitution of the amino acid glycine for arginine at nucleotide 16) which is associated with augmented vasodilator responses to β-adrenergic agonists in skeletal muscle vascular beds. In addition, this augmented responsiveness appears to be dependent on the NO component of β-adrenergically mediated vasodilation, as differences between the two polymorphisms are absent after NO synthase inhibition (16). As these polymorphisms exist in a Hardy-Weinberg equilibrium, roughly one quarter of the population are likely to be homozygous for the more responsive (and more NO-dependent) β_2-adrenergic receptor. As such, this population may include individuals with the most pronounced vasodilator responses to hypoxia. This is an exciting area for further study.

SLEEP APNEA

Hypoxic vasodilation may be altered by overlying pathophysiological conditions such as sleep apnea. Remsburg *et al.* (32) have shown that the vascular response to hypoxia in patients with sleep apnea is vasoconstriction, as opposed to the vasodilation seen in healthy subjects. An obvious question in the context of this study is whether sleep apnea patients have a reduced vasodilator signal during hypoxia (e.g., less epinephrine?), or an augmented sympathetic vasoconstrictor response. In other words, the vasodilator-vasoconstrictor balance is shifted in individuals chronically exposed to periodic nocturnal asphyxia so as to favor vasoconstriction over the normal vasodilator response. It is unclear whether or not this observation is linked to the prevalence of hypertension in this patient group.

ORTHOSTASIS AND HYPOXIC SYNCOPE

The many factors that determine skeletal muscle vascular tone during hypoxic stress impact on the regulation of arterial pressure. Early work by Henderson *et al.* (21)and Anderson *et al.* (1) demonstrated that vasovagal-like syncope could be produced in most individuals by having them breathe low O_2 levels (< 8 %). These studies may be the earliest documentation that hypoxia can have profound influences on cardiovascular regulation in humans. Surprisingly, these early studies found that some individuals will become syncopal while breathing only moderately hypoxic O_2 mixtures (13 – 14 % O_2) (21), similar to the O_2 levels in the natural environment at altitudes of 3000-4000 m. It should be noted that these vasovagal responses occurred in supine subjects. The effect is more striking (and occurs at more modest levels of hypoxia) in upright subjects. More recent studies have documented reduced tolerance to orthostatic stress at altitude (4000 m) and during hypoxic breathing at sea level that simulated altitude (2500-4300 m) (28, 37, 38, 29). These cases of hypoxic syncope are clearly differentiated from the effects of central nervous system hypoxia (hypoxic coma). Hypoxic syncope appears to be a form of vasovagal syncope (i.e., vasodilation, bradycardia, and hypotension have been observed), from which an individual can recover spontaneously. In contrast, profound central nervous system hypoxia leads to a depression of higher center functioning, leading to stupor and subsequent coma, without concomitant vasovagal signs (1, 21). The incidence rate of hypoxic syncope among visitors to altitude remains unknown, but is probably significant.

Early hemodynamic studies highlighted a potential role of exaggerated circulating epinephrine levels in precipitating hypoxic syncope (34, 45). Figure 7 shows two examples of hypoxic syncope that occurred during ongoing studies. In one case, (Figure 7, panel B) an arterial blood sample was collecting as the subject became hypotensive and bradycardic. Results were consistent with this notion of exaggerated circulating epinephrine (4-fold higher than other subjects exposed to the same degree of hypoxia)(14). High epinephrine was associated with progressive skeletal muscle vasodilation, but part of the hypotension may also be linked to hypoxic vasodilation of the splanchnic circulation (34, 45). In further support of this "working hypothesis", one case report suggests that β-adrenergic blockade may prevent hypoxic syncope. (15)

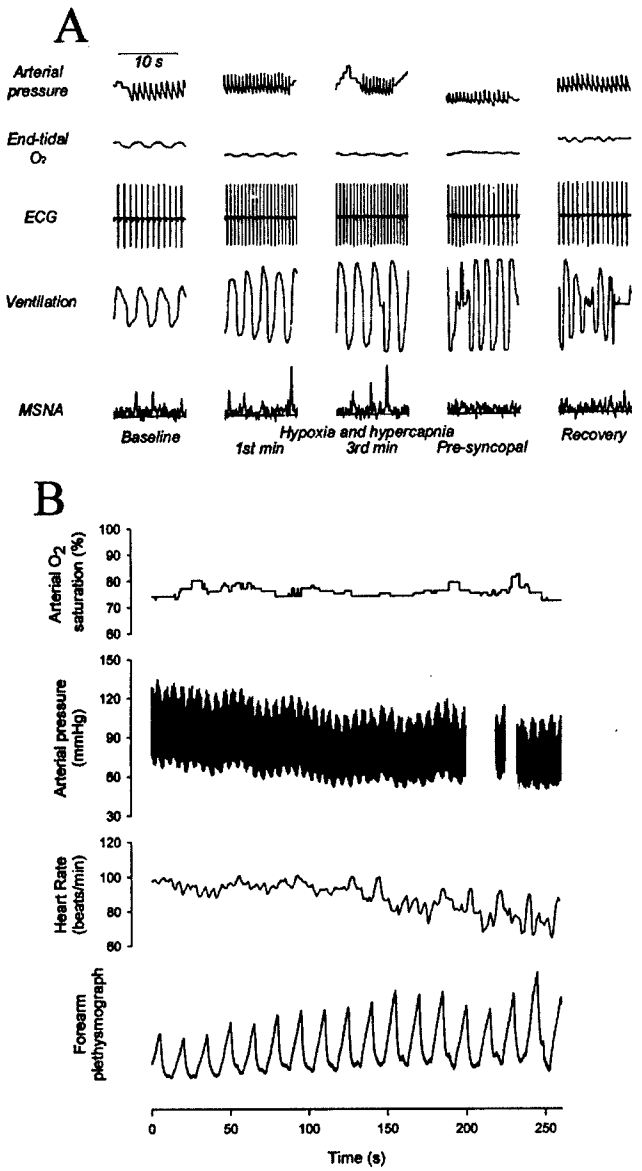

Figure 7. Two examples of hypoxic syncope from routine laboratory investigations. A: A supine subject developed bradycardia, hypotension, and pre-syncopal symptoms after a few minutes of combined hypoxia and hypercapnia. Notice the profound sympathoinhibition during the pre-syncopal time period, consistent with the classic vasovagal response. Tracing courtesy of Barbara J. Morgan (University of Wisconsin). B: A supine subject who developed bradycardia, hypotension, and pre-syncopal symptoms after a few minutes of isocapnic hypoxia. Progressive skeletal muscle vasodilation is documented by the forearm plethysmography tracing. An arterial blood sample drawn near the end of the response revealed a surge of epinephrine (from 54 to 340 pg/ml) that coincided with the vasovagal response. (Halliwill & Dinenno, unpublished observations)(14).

SUMMARY

As summarized in Figure 6, we now have a "working model" for hypoxic vasodilation in skeletal muscle vascular beds in humans. A clear role has been demonstrated for increased activity of both β-adrenergic and α-adrenergic receptors, in opposition to one another, during exposure to hypoxia. Strong evidence also supports a role for adenosine or a related compound, perhaps functioning through ATP-sensitive K^+-channels. It is also thought that NO from the endothelium may serve as a necessary component or final signal for some of these pathways, but further studies are needed to fully define and understand this role. Finally, current studies are investigating the possibility that α-adrenergic pathways are modified during hypoxia (i.e., functional sympatholysis)(13) and the extent to which this model is relevant to other vascular beds in humans (e.g., cutaneous vasculature)(27).

ACKNOWLEDGEMENTS

I would like to thank my colleagues, Drs. Christopher T. Minson and Frank A. Dinenno, for contributing important suggestions and sharing my ongoing interest in this work. This work was supported in part by a grant from the Wilderness Medical Society (Herbert N. Hultgren Award) and National Institutes of Health (NIH) Grant HL-65305.

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Chapter 17

HYPOXIC REGULATION OF BLOOD FLOW IN HUMANS

α-adrenergic receptors and functional sympatholysis in skeletal muscle

Frank A. Dinenno

Abstract: Acute exposure to hypoxia evokes changes in local vasodilator and neural vasoconstrictor factors that significantly influence vascular tone. In humans, the net effect of acute systemic hypoxia is limb vasodilation despite significant reflex increases in muscle sympathetic vasoconstrictor nerve activity and norepinephrine spillover. In this context, some studies in experimental animals and humans have documented that hypoxia can reduce the vasoconstrictor responses to sympathetic nerve stimulation, as well as exogenous α-adrenergic agonist administration (functional sympatholysis). In contrast, other studies have provided evidence that sympathetic vasoconstriction is well preserved during hypoxia. Recently, our laboratory demonstrated that local blockade of α-adrenergic receptors significantly augments the forearm vasodilator response to hypoxia, indicating that sympathetic vasoconstriction persists and can restrain skeletal muscle blood flow under these conditions. Therefore, we revisited this issue and performed a study designed to test the hypothesis that forearm vasoconstrictor responses to local endogenous norepinephrine release are not reduced during systemic hypoxia in humans. To do so, we used selective intra-arterial infusions tyramine to evoke local endogenous norepinephrine release and measured the forearm vasoconstrictor responses during various levels of hypoxia (85, 80, and 75 % O_2 saturation). Our findings demonstrate that forearm post-junctional α-adrenergic vasoconstrictor responsiveness is well preserved during systemic hypoxia in healthy humans. The implications of these findings with respect to arterial blood pressure regulation and functional sympatholysis in skeletal muscle are discussed.

Key Words: muscle blood flow, sympathetic vasoconstriction, metabolic inhibition

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

INTRODUCTION

It is well known that sympathetic α-adrenergic vasoconstrictor responses can be altered by changes in the local metabolic milieu of skeletal muscle. Specifically, increases in local metabolism via muscle contractions (5, 27, 39, 41) and/or reductions in the tissue PO_2 (hypoxia) (23, 35, 37) and pH levels (acidosis) (24, 38) can inhibit or blunt sympathetic vasoconstriction (functional sympatholysis), facilitating metabolic regulation of muscle blood flow and oxygen delivery under these conditions. Studies in the rat microcirculation suggest that this "metabolic inhibition" of vasoconstrictor responses occurs primarily via post-junctional α_2-adrenergic receptors located in the smallest resistance vessels in close proximity to skeletal muscle fibers, whereas the vasoconstriction mediated via α_1-receptors (in larger upstream vessels) is relatively preserved (1, 24, 39). This control of muscle blood flow has been hypothesized to serve two primary functions: (1) local metabolic inhibition of α_2 receptors to promote adequate blood flow and oxygen delivery, and (2) maintained α_1 vasoconstriction in larger vessels to regulate regional vascular resistance and ultimately, arterial blood pressure.

In humans, acute systemic hypoxia evokes significant changes in the local, humoral, and neural determinants of vascular tone (see Figure 6 of preceeding chapter). The roles of the various vasodilator substances that contribute to skeletal muscle vasodilation during hypoxia are discussed in a companion review by Halliwill (13). With respect to the latter, muscle sympathetic vasoconstrictor nerve activity increases during hypoxia (32, 34) without significant changes in arterial or venous plasma norepinephrine concentrations. Although this finding could reflect an increase in the clearance of norepinephrine, Leuenberger *et al.* demonstrated that this increase in sympathetic outflow does result in an increase in norepinephrine spillover (22). Despite these elevations in sympathetic vasoconstrictor activity and subsequent norepinephrine spillover, limb vasodilation is observed and is graded with the level of hypoxia (18, 28). Thus, it appears as though systemic hypoxia "uncouples" the elevated sympathetic activity from the predicted end organ response (vasoconstriction). As such, many investigators often assume that the vasoconstriction mediated via post-junctional α-adrenergic receptors in response to endogenous norepinephrine release is blunted (functional sympatholysis) in the vascular beds of skeletal muscle during hypoxia. However, data derived from experimental animals and humans have both supported (18, 23, 35, 37) and refuted (10, 15, 17, 31, 33) this hypothesis.

In a recent study, our laboratory demonstrated that the forearm vasodilator responses to hypoxia are significantly augmented (~2 fold) after local blockade of α-adrenergic receptors (43), implicating that sympathetic vasoconstriction persists and restrains skeletal muscle blood flow under these conditions. This finding raises questions as to whether sympathetic vasoconstrictor responses in human skeletal muscle are truly blunted during systemic hypoxia. Therefore, using intra-arterial infusions of tryamine to evoke local endogenous norepinephrine release and subsequent α_1- and α_2-adrenergic receptor stimulation, we recently revisited this issue (9) and tested the hypothesis that sympathetic neural vasoconstriction is not blunted during systemic hypoxia in healthy humans.

OVERVIEW OF METHODS

A total of 18 young healthy adults participated in the study. The specific details of the methods employed and experimental protocol is presented elsewhere (9). A 20-gauge, 5-cm catheter was placed in the brachial artery of the non-dominant arm for measurement of arterial pressure and local tyramine administration (6), and an 18-gauge 3-cm catheter was inserted in retrograde fashion in a deep antecubital vein that drained the forearm muscles (20). Blood samples were obtained from the brachial artery and analyzed with a clinical blood gas analyzer (Bayer 855 Automatic Blood Gas System, Boston, MA, USA) for partial pressures of O_2 and CO_2 (PO_2 and PCO_2), pH and hemoglobin O_2 saturation. Additionally, brachial artery plasma catecholamine (epinephrine and norepinephrine) and deep venous norepinephrine concentrations were determined by HPLC with electrochemical detection (7, 25). Forearm blood flow (FBF) was estimated simultaneously in both the control and experimental (catheterized) limb by venous occlusion plethysmography with mercury-in-silastic strain gauges (12), and forearm vascular conductance (FVC) was calculated as (FBF x 100)/MAP, and expressed as arbitrary "units."

Hypoxia was achieved by manipulating the level of O_2 provided in the inspiratory gas by mixing N_2 with air via a medical gas blender. We employed a self-regulating partial-rebreathe system developed by Banzett *et al.* to maintain constant alveolar fresh-air ventilation independent of changes in breathing frequency or tidal volume (2, 43) and to clamp end-tidal CO_2 levels despite large changes in minute ventilation in response to hypoxia. In the first 10 subjects, the level of O_2 was titrated down to achieve an arterial O_2 saturation of 85% as assessed by pulse oximetry of the earlobe, whereas in the following 8 subjects this was titrated to achieve an O_2 saturation of 85, 80, and 75%.

In 8 men and 2 women, forearm vasoconstrictor responses to tyramine (2 and 8 μg (dl forearm volume)$^{-1}$ minute^{-1}) were assessed during normoxia and mild systemic hypoxia. In 5 men and 3 women, forearm vasoconstrictor responses to tyramine (8 μg (dl forearm volume)$^{-1}$ minute^{-1}) were assessed during normoxia and the graded levels of hypoxia (85, 80, and 75% O_2 saturation) to determine whether a threshold level of hypoxia was necessary to blunt sympathetic vasoconstriction. We chose tyramine as our method of sympathetic "activation" because it evokes endogenous norepinephrine release (11), which subsequently stimulates α_1- and α_2-adrenergic receptors normally stimulated by increases in sympathetic nerve discharge (19). Thus, it allows the study of the vasoconstrictor effects of local norepinephrine release during hypoxia without the possible confounding influences of other forms of sympathoexcitation (e.g., lower body negative pressure) that might evoke changes in vasoactive substances not associated with hypoxia *per se* and possibly vasovagal responses (30). Further, tyramine does not have any direct vasoconstrictor effects (11), and the vascular responses to tyramine are abolished by non-selective α-adrenergic blockade (7, 8).

RESULTS

In general, hemoglobin O_2 saturation and PO_2 decreased progressively with the target level of hypoxia, and ventilation and heart rate significantly increased. PCO_2, pH, and MAP were similar in all trials. The increases FBF and FVC in response to mild and graded

levels of hypoxia were similar in both the control and experimental arms and ranged from ~20-50%. Consistent with the findings of previous studies, arterial norepinephrine concentrations were similar during normoxia and all levels of hypoxia, whereas arterial epinephrine concentrations increased with the severity of hypoxia.

The percentage reductions in FBF and FVC to both doses of tyramine during mild hypoxia were not significantly different than during normoxia (Figure 1A). Similarly, the vasoconstrictor responses were well preserved during the graded levels of hypoxia (Figure 1B). Given that hypoxia increased baseline FBF and FVC, the absolute reductions in FBF and FVC tended to be greater during hypoxia. The tyramine-evoked changes in deep venous norepinephrine concentrations were not different during normoxia and hypxoxia (Figure 2), indicating that post-junctional α-adrenergic responsiveness is not blunted during systemic hypoxia. Control limb hemodynamics were not affected by tyramine administration in the experimental arm.

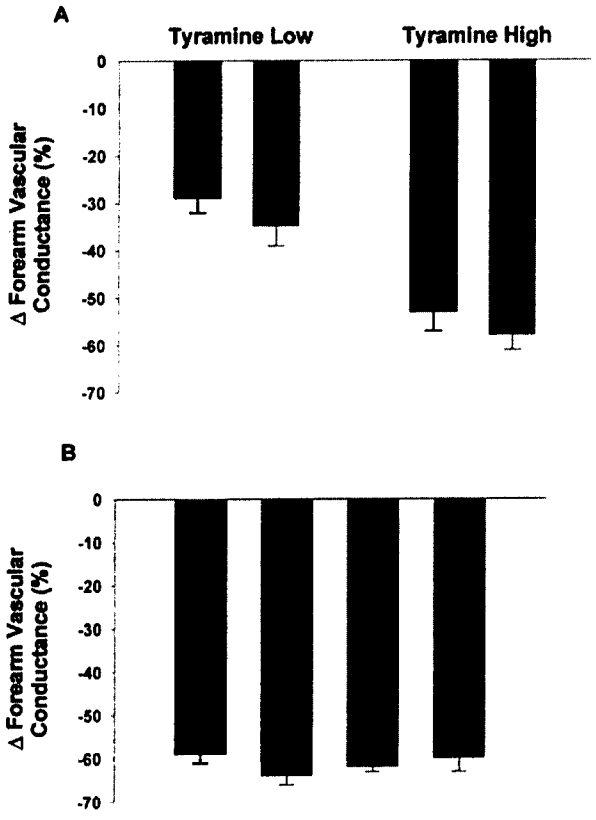

Figure 1. The forearm vasoconstrictor responses to both low and high doses of tyramine were not different during normoxia (black bars) and when O_2 saturation was reduced to 85%(mild hypoxia – grey bars; A). Additionally, in another group of subjects, the vasoconstrictor responses to the high dose of tyramine were well preserved when O_2 saturation was reduced down to 75% (B). Adapted from reference 9.

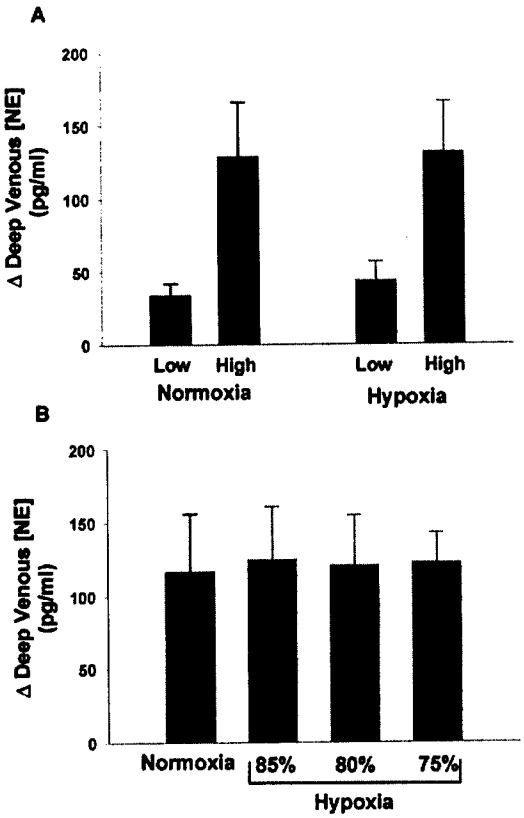

Figure 2. Low and high doses of tyramine evoked similar increases in deep venous norepinephrine (NE) concentrations during both normoxia and mild hypoxia (85% $\dot{O}_2$ saturation; A). These increases were also similar during normoxia and hypoxia in those subjects who underwent the graded levels of hypoxia (B). Thus, the preserved vasoconstrictor responses presented in Figure 1 indicate that post-junctional α-adrenergic vasoconstrictor responsiveness is not blunted during systemic hypoxia in healthy humans. Adapted from reference 9.

DISCUSSION

Whether sympathetic α-adrenergic vasoconstriction is blunted in the vascular beds of skeletal muscle during acute systemic hypoxia has been a topic of considerable debate. In a recent study, we demonstrated that the forearm vasodilator responses to systemic hypoxia were augmented after local blockade of α-adrenergic receptors (43), suggesting that sympathetic vasoconstriction persists and can restrain limb blood flow under these conditions in humans. Thus, it was our goal to revisit this issue utilizing a novel approach to study the local vascular responses to neurally-released norepinephrine (via intra-arterial tyramine) during various levels of hypoxia. Given that other sympathoexcitatory manuevers during hypoxia can evoke vasovagal responses in some subjects and/or changes in other vasoac-

tive factors not associated with hypoxia *per se*, we feel that our approach reduces (if not eliminates) these confounds with respect to the interpretation of sympathetic vasoconstrictor responses during hypoxia.

Our findings clearly demonstrate that the ability of post-junctional α-adrenergic receptors to respond and evoke vasoconstriction to neurally-released norepinephrine is well preserved during systemic hypoxia in conscious humans. This observation is consistent with some (15, 31, 33), but not all (18), studies in humans. Studies in experimental animals have also yielded equivocal results (10, 17, 23, 35, 38). Although it is not entirely clear why there have been such discrepant findings, the methods of sympathetic activation in humans (e.g., lower body negative pressure or exogenous norepinephrine infusions) and levels of hypoxia achieved in both the animal and human studies might have played a role. In this context, some studies in animals have determined sympathetic vasoconstriction at levels of tissue hypoxia that are impossible to achieve during systemic hypoxia in humans (e.g., PO_2 ~5 mmHg). Nevertheless, our findings are consistent with more recent data demonstrating preserved forearm vasoconstriction to sympathetic activation during systemic hypoxia in healthy humans (15, 33).

Hypoxia And Orthostatic Tolerance

It has long been postulated that hypoxia impairs blood pressure regulation, increasing the incidence of orthostatic intolerance. Alterations in sympathetic nervous system function such as impairments in norepinephrine release or baroreflex modulation of muscle sympathetic nerve activity, as well as reductions in α-adrenergic vasoconstrictor responsiveness, could potentially lead to impairments in blood pressure regulation. However, in contrast to original theories with respect to reduced norepinephrine release during hypoxia, recent studies have demonstrated that the elevated muscle sympathetic activity does indeed evoke norepinephrine release during hypoxia (22). Additionally, Halliwill and Minson have demonstrated that sympathetic-baroreflex gain is not impaired during systemic hypoxia in humans (14). Further, the results of our study demonstrate that post-junctional α-adrenergic responsiveness is not blunted (9). Thus, it does not appear that there are major deficits in baroreflex-mediated increases in sympathetic outflow during reductions in arterial blood pressure, or reductions in end organ responsiveness (α-adrenergic vasoconstriction) during hypoxia in healthy humans. Taken together, these recent data support the hypothesis that peripheral vasoconstrictor responses and arterial blood pressure regulation are not impaired in most subjects during hypoxia, and that vasovagal syncope (sudden bradycardia and sympathetic withdrawal) occurs in some subjects that demonstrate drastic increases in circulating epinephrine (30). It is very interesting to note the one subject in our recent study who was unable to complete the 75% O_2 saturation trial demonstrated an exaggerated rise in arterial plasma epinephrine concentrations (from 54 to 340 pg • ml^{-1}), as well as bradycardia (Δ HR = -19 beats min^{-1}) and hypotension (Δ MAP = -22 mmHg). Although the factors that determine whether an individual becomes syncopal during hypoxia are not entirely understood, drastic increases in circulating epinephrine appear to be mechanistically-linked to vasovagal syncope.

Hypoxia And Functional Sympatholysis In Skeletal Muscle: Insights From Muscle Contractions

In 1962, Remensnyder *et al.* demonstrated that the vasoconstrictor responses to sympathetic nerve stimulation are significantly blunted in the vascular beds of contracting compared with resting dog skeletal muscle (27). This observation, termed "functional sympatholysis", has subsequently led to numerous experiments that have both challenged and supported this concept. However, more recent studies in both animals (1, 5, 39) and humans (16, 41) have clearly provided experimental evidence that muscle contractions can blunt sympathetic vasoconstriction. Figure 3 illustrates the ability of muscle contractions to blunt sympathetic neural vasoconstriction in humans (41). Although the mechanisms underlying this phenomenon have not been fully elucidated, it has been hypothesized that tissue hypoxia (15) and/or acidosis (24), as well as newly synthesized nitric oxide (40) might be responsible for interfering with sympathetic vasoconstriction in contracting skeletal muscle, facilitating metabolic regulation of blood flow.

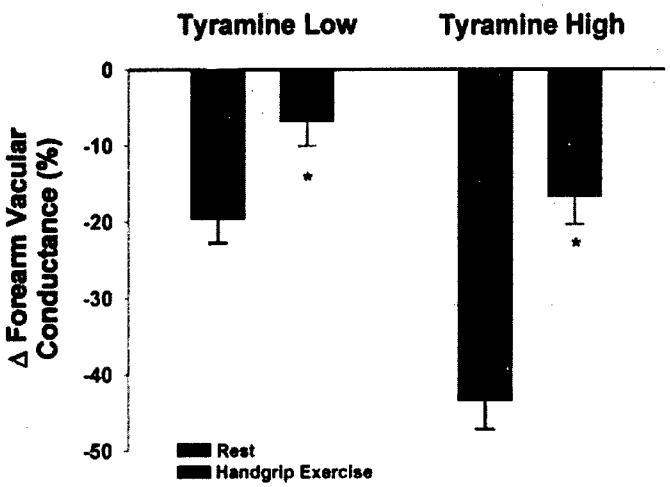

Figure 3. Tschakovsky *et al.* demonstrated that the forearm vasoconstrictor responses to the same doses of tyramine used in our systemic hypoxia protocol are significantly blunted during rhythmic handgrip exercise (20-25% maximum voluntary contraction) compared with rest. This supports the concept of functional sympatholysis in the vascular beds of contracting skeletal muscle in humans, and is in contrast to our findings during systemic hypoxia. Modified from reference 41.

The results of our study demonstrate that systemic hypoxia does not blunt the vasoconstrictor responses to endogenous norepinephrine release, but certainly does not rule out the possibility that local tissue hypoxia achieved via muscle contractions can reduce this vasoconstriction. In this context, Hansen and colleagues (15) recently determined the interactions between hypoxia and muscle contraction in mediating the blunted vasoconstrictor responses to sympathetic stimulation observed during exercise. These investigators demonstrated that when subjects breathed a 10% O_2 gas mixture, the ability of sympathetic

activation to evoke vasoconstriction was not impaired, in accordance with our findings. However, when hypoxia and mild muscle contraction (5% maximum voluntary contraction; MVC) were performed simultaneously, the vasoconstrictor responses were abolished, indicating that local tissue hypoxia might be causally-linked to functional sympatholysis during exercise. If tissue hypoxia is indeed involved in this process, future studies will be needed to determine what the critical tissue PO_2 is necessary to inhibit the vasoconstrictor responses to norepinephrine in humans.

Given that we did not observe any blunting of α-adrenergic responsiveness to endogenous norepinephrine release in this study, we did not "pharmacodissect" the α-receptor subtypes. However, whether or not metabolic inhibition of sympathetic vasoconstriction during exercise occurs primarily via post-junctional α_2-adrenergic receptors in humans (as suggested from studies in animals) has been recently investigated in our laboratory. Using selective intra-arterial infusions of α_1- and α_2-adrenergic agonists, we demonstrated that the vasoconstrictor responses mediated via both receptor subtypes are significantly blunted during moderate rhythmic handgrip exercise (~10-15% MVC; Figure 4), with no apparent difference in the magnitude of inhibition between α_1 and α_2 receptors (29). This is in stark contrast to the data derived from experimental animals suggesting that α_2-adrenergic responsiveness is blunted during all levels of exercise and that α_1-responsiveness is blunted only at heavy workloads (1, 5). It is important to note that at the exercise intensity employed in our study, it is very unlikely that any tissue hypoxia and/or acidosis occurred (21, 36). Thus, it appears that tissue hypoxia and/or acidosis might not be obligatory to reduce sympathetic vasoconstriction in active skeletal muscle. These new data raise questions not only about the mechanisms involved in functional sympatholysis in humans, but also raise questions about the distribution of α_1- and α_2-adrenergic receptors in skeletal muscle resistance vessels of humans (i.e., larger vs smaller vessels).

Implications For Skeletal Muscle Vasodilation During Systemic Hypoxia

One obvious question we are left with regarding the integrative control of muscle blood flow during systemic hypoxia follows: if sympathetic vasoconstriction is preserved during hypoxia in humans, then how does muscle blood flow and vascular conductance increase when there are significant elevations in sympathetic vasoconstrictor activity? As discussed in a companion review by Halliwill (13), there appears to be a role for epinephrine, adenosine, prostaglandins, as well nitric oxide in the skeletal muscle vasodilator response to hypoxia (3, 4, 26, 43). Although these factors directly relax vascular smooth muscle, it is also possible (as suggested by Vanhoutte) that increasing concentrations of local factors inhibit norepinephrine release at low levels of sympathetic nerve activity, but not as the rate of sympathetic nerve firing increases (42). If this were the case, this could explain the observations of initial hypoxia-induced skeletal muscle vasodilation that still remains under the influence of sympathetic vasoconstrictor tone as sympathetic activity increases (43). We speculate that once the net effect of these factors have resulted in the appropriate elevation in blood flow and oxygen delivery, then further sympathetic activation and subsequent norepinephrine release can evoke normal α-adrenergic vasoconstrictor responses.

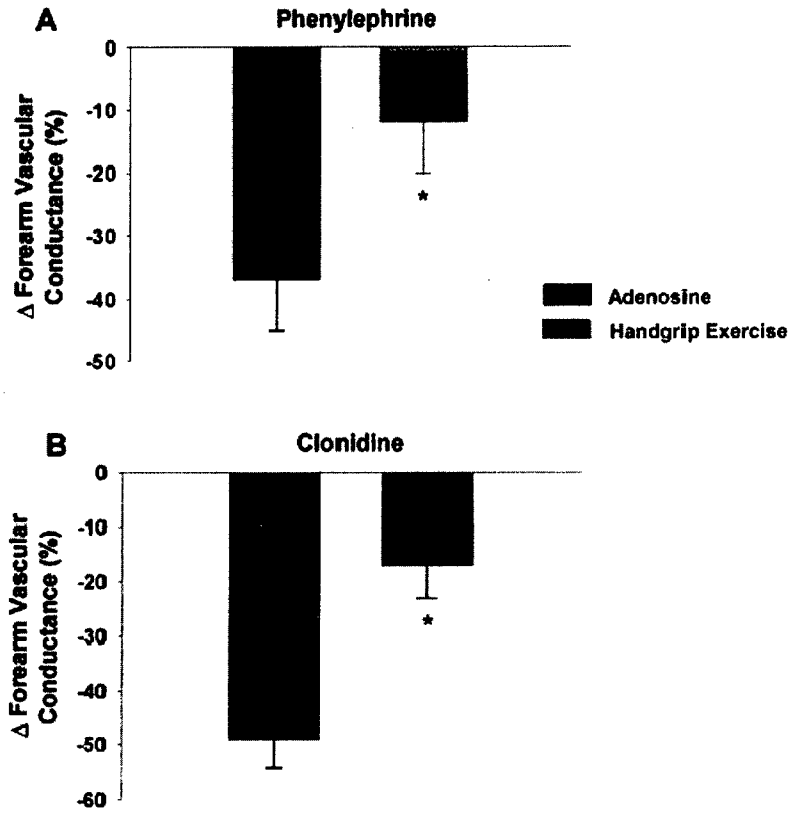

Figure 4. Recent data from our laboratory demonstrating that the vasoconstrictor responses to the α_1-adrenergic agonist phenylephrine (A) and the α_2-agonist clonidine (B) are significantly blunted during moderate rhythmic handgrip exercise compared with a control vasodilator condition (intra-arterial adenosine) in humans. This is in contrast to data derived from experimental animals indicating that post-junctional α_2-receptors are much more sensitive to metabolic inhibition especially at light workloads. Adapted from reference 29.

CONCLUSIONS

The results from our recent study demonstrate that post-junctional α-adrenergic vasoconstrictor responsiveness to endogenous norepinephrine release is not blunted in the forearm during systemic hypoxia in humans (9). However, it is important to note that this does not rule out a possible role for local tissue hypoxia as a key process involved in the blunted vasoconstrictor responses observed during muscle contractions. Finally, recent findings from our laboratory indicate that both α_1- and α_2-adrenergic vasoconstrictor responsiveness are blunted during mild exercise in humans. This latter finding is interesting because it is unlikely that overwhelming tissue hypoxia or acidosis occurs at this level of exercise, raising questions about the mechanisms involved in functional sympatholysis in humans.

Finally, the distribution of α_1- and α_2-adrenergic receptors in skeletal muscle resistance vessels might differ from that of experimental animals.

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Chapter 18

HYPOXIC REGULATION OF BLOOD FLOW IN HUMANS

Skin blood flow and temperature regulation

Christopher T. Minson

Abstract: Regulation of cutaneous vascular tone in humans is complex due to the different types of skin in various regions of the body and the vast array of nerves involved in regulation of blood flow. Due to these complexities, it is unclear how the cutaneous vasculature responds to hypoxia. There are reports of exaggerated vasoconstriction and vasodilation, while others suggest the skin is unresponsive to a hypoxic stimulus. Preliminary work in our laboratory suggests hypoxic vasodilation may be unmasked with α-receptor blockade. In contrast to skeletal muscle, hypoxic cutaneous vasodilation is not blunted by β-blockade, but may be abolished with NO-synthase inhibition. Furthermore, effects of hypoxia on skin blood flow may be more pronounced during combined hypoxic and thermoregulatory challenges. Along these lines, overall thermoregulation may be impacted by hypoxic effects on the cutaneous vasculature and hypobaric effects on sweating and evaporation. During supine heat stress, for example, skin blood flow can increase to 8 Liters per minute. This dramatic rise in skin blood flow is accomplished by an increase in cardiac output and redistribution of blood flow from the splanchnic and renal vascular beds. During hypoxia, splanchnic blood flow has been shown to increase. Thus, during a hypoxic challenge in the heat, a competition for blood flow between the compliant skin and splanchnic regions must exist, but is not well understood. In this review, the effects of hypoxia on the regulation of cutaneous vascular tone and the impact on temperature regulation will be discussed.

Key Words: cutaneous, thermoregulation, acral, glabrous, exercise

INTRODUCTION

Regulation of blood pressure and distribution of blood flow in response to hypoxic conditions involves reflex control of the regional circulations. The importance of a given

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

circulation in meeting such regulatory challenges is directly related to the fraction of the total vascular conductance in that region, as well as in the degree of vasoconstriction or vasodilation attending the challenge. In resting thermoneutral conditions, skin blood flow accounts for less than 10% of total vascular conductance. The relatively small changes in blood flow to the skin observed in a hypoxic environment probably do not significantly impact overall cardiovascular regulation. However, even a small increase in skin blood flow can lower core body temperature by increasing the temperature gradient between skin and the environment. Moreover, the absolute amount of blood flow to the skin can increase during heat stress to as much as 8 liters per minute. This dramatic increase in skin blood flow is met by an increase in cardiac output and redistribution of blood flow from other regions, particularly the splanchnic and renal vascular beds. In this setting, the cutaneous circulation can comprise over 60% of total vascular conductance, greatly challenging blood pressure regulation and impacting blood volume distribution throughout the circulation. Exposure to a hypoxic environment also causes complex changes in regulation of systemic hemodynamics and blood pressure (see accompanying reviews, (7, 16). Thus, the potential for hypoxia to alter the regulation of cutaneous vascular tone or for thermoregulatory reflexes to impact vascular regulation and blood volume distribution to a hypoxic challenge exists, yet remains poorly understood. The goal of this manuscript is to review the current literature investigating the influence of hypoxia on regulation of cutaneous vascular tone, discuss how thermoregulatory responses may be altered in a hypoxic environment, and identify specific areas where more research is needed.

HUMAN SKIN

Most of the body surface area is covered with so-called "hairy" or non-acral skin (also called "non-glabrous" skin), whereas the skin of the lips, ears, nose, palms of the hands and fingers, and plantar aspects of the feet are acral skin (also referred to as "glabrous" skin). In total, the skin covers about $1.8m^2$ and accounts for approximately 5% of total body weight in humans (24). The skin of humans consists of two layers: a superficial layer, the epidermis, and a deep layer, the dermis. The epidermis is almost entirely comprised of keratinized squamous epithelial cells, whereas the dermis has a more complex histology and contains blood vessels, afferent and efferent nerves, sebaceous glands, sweat glands, and hair follicles. Most blood vessels in the dermis are found in the papillary plexus, which is made up of high-resistance arterioles, papillary loops, and postcapillary venules. The papillary loops are located close to the dermal-epidermal junction, so the temperature gradient from blood to epidermal tissue is great. This temperature gradient favors heat exchange between the blood and the external environment. In addition, the surface area of the papillary loops is very large, so regulation of blood flow through the papillary loops by the arterioles can greatly impact heat exchange between the body's core and the environment.

REGULATION OF BLOOD FLOW IN NON-ACRAL SKIN

In non-acral skin, two branches of the sympathetic nervous system control blood flow: a vasoconstrictor system and an active vasodilator system. The vasoconstrictor system

is adrenergic, releasing norepinephrine binding to post-junctional α_1- and α_2-adrenergic receptors. The mechanisms of active vasodilation are less-well understood. The prevailing theory suggests that these nerves are sympathetic cholinergic (or sudomotor) nerves, releasing acetylcholine, and are the same nerves that innervate sweat glands. During whole body heating sufficient to raise core body temperature, there is an increase in skin blood flow at about the same time that sweating begins, suggesting a possible linkage of the two. However, blockade of muscarinic receptors in the skin with atropine abolishes sweating, but has little influence on the rise in skin blood flow during heat stress. In contrast, presynaptically blocking release of neurotransmitters in these cholinergic nerves by injecting botulinum toxin to an area of skin inhibits both sweating and active vasodilation (21). Botulinum toxin is an anticholinergic agent that abolishes release of acetylcholine and any colocalized neurotransmitters from cholinergic nerves. These data suggest that acetylcholine controls sweating via activation of muscarinic receptors, and a cotransmitter released with acetylcholine mediates active vasodilation.

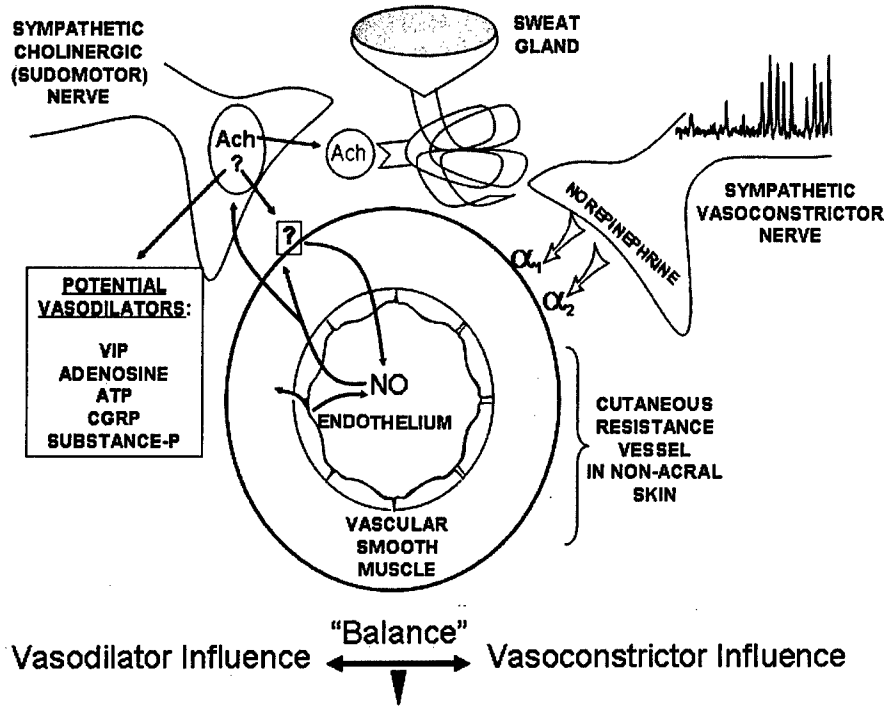

Figure 1. Neural control of non-acral skin blood flow. Non-acral skin blood flow is controlled by two branches of the sympathetic nervous system: a vasoconstrictor system and an active vasodilator system. The vasoconstrictor system is adrenergic, releasing norepinephrine binding to post-junctional α_1- and α_2-adrenergic receptors. The active vasodilator system is not well understood. The prevailing theory suggests that acetylcholine (Ach) and an unknown neurotransmitter are co-localized and co-released from sympathetic cholinergic nerves. Nitric oxide (NO) also has a role in active vasodilation by directly mediating a portion of vasodilation and interacting with the unknown vasodilator at the level of the second messenger system or by affecting neuronal release.

A second theory for active vasodilation suggests that sweating and active vasodilation may be controlled by two separate nerves. Evidence for this theory comes from the observation that combining isometric handgrip exercise during hyperthermia causes vasoconstriction of the cutaneous arterioles, but an increase in sweat rate in non-acral skin (4). This was also observed in an area of skin in which bretyllium tosylate was applied, which inhibits the release of norepinephrine from adrenergic nerves. This finding implies the relative vasoconstriction observed during isometric handgrip was due to withdrawal of active vasodilation, despite an increase in sweat rate. However, if active vasodilation and sweating are mediated by different nerves as this theory suggests, both nerves are sensitive to presynaptic blockade with botulinum toxin. Withdrawal of active vasodilation during isometric handgrip exercise in a hyperthermic state brings up an important point. That is, when maneuvers causing cutaneous vasoconstriction in normothermia are performed during heat stress, active vasodilation is withdrawn, as opposed to a superimposed increase in vasoconstrictor tone (18).

The list of potential neurotransmitters for the active vasodilator substance includes vasoactive intestinal peptide (VIP), adenosine, ATP, calcitonin gene-related peptide (CGRP), and substance P (SP). These vasoactive substances have been shown in various studies to be present in the skin and to induce vasodilation (17, 42, 47). Current evidence suggests that CGRP and SP are the primary neurotransmitters of neurogenic inflammation, axon reflexes, and pain sensation in the skin (42). VIP, on the other hand, has been found in cholinergic nerves innervating sweat glands in human skin (17, 41). Thus, a role for VIP in active vasodilation appears likely, and is an area of focus in our laboratory.

Because NO can be released by non-adrenergic vasodilator nerves, and by the vascular endothelium in response to neural or mechanical stimulation, a role for NO in mediating active cutaneous vasodilation has been suggested. Recent studies in humans have shown quite convincingly that active cutaneous vasodilation in humans requires functional NO-synthase to achieve full expression, but that NO is not the unknown vasodilator substance (20, 43, 44). Specifically, NO-synthase inhibition attenuates reflex vasodilation in response to body heating by ~30% in humans. Recent work in our laboratory has further determined that NO acts "synergistically" with the unknown vasodilator (51). That is, NO has a direct vasodilator role in active vasodilation, but also potentiates the vasodilator action of the neurotransmitter. Thus, NO may serve as a site for regulation of active vasodilation, and factors that affect production of NO, such as hypoxia, may impact cutaneous vascular tone during hyperthermia via this mechanism.

SYSTEMIC RESPONSES TO INCREASED SKIN BLOOD FLOW

Although skin blood flow totals 300-500 milliliters per minute in resting thermoneutral conditions, the absolute amount of skin blood flow can vary from nearly zero during periods of maximal vasoconstriction to as much as 8 Liters per minute during heat stress (33). This increase in skin blood flow is achieved by an increase in cardiac output and redistribution of blood flow from other areas, particularly the splanchnic, renal, and skeletal muscle vascular beds. As skin is a very compliant circulation, an increase in skin blood flow results in a large peripheral displacement of blood volume and a resulting decrease in central venous pressure (38). The splanchnic vascular bed is also very compliant, such that

a rise in skin blood flow typically is accompanied by a concomitant decrease in splanchnic blood flow (36). Thus, reflex control of the cutaneous arterioles can greatly impact systemic hemodynamics during hyperthermia. Alternatively, factors that impact blood flow distribution throughout the circulation, such as hypoxia or exercise, can profoundly alter skin blood flow.

HYPOXIA AND REGULATION OF NON-ACRAL SKIN BLOOD FLOW

The primary determinant of skin blood flow in non-acral skin is core temperature. During resting, thermoneutral conditions the skin is under tonic vasoconstrictor influence. As core temperature decreases, vasoconstriction is augmented. As core temperature increases, withdrawal of vasoconstrictor tone occurs, resulting in an approximate doubling of skin blood flow. A further increase in core temperature above a "threshold temperature" initiates active vasodilation and sweating. Both the threshold of thermoregulatory responses and the slope or sensitivity of the responses (versus core temperature) can be influenced by a number of factors.

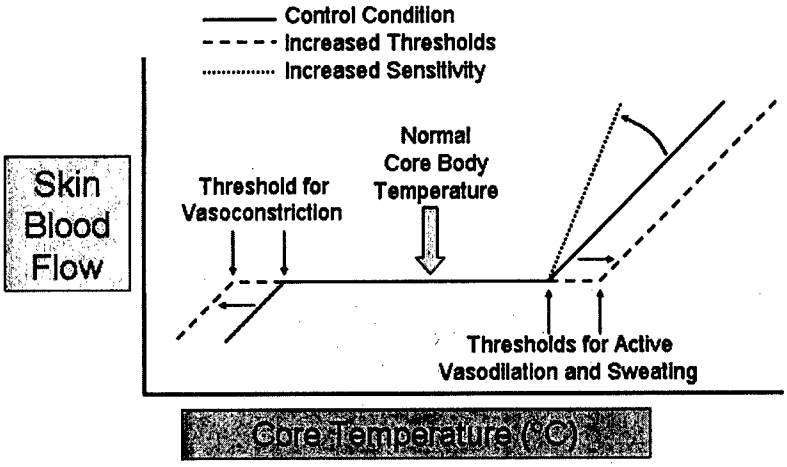

Figure 2. Threshold and sensitivity changes in thermoregulatory responses. Core temperature is the primary determinant of thermoregulatory responses. A number of factors, including hypoxia and skin temperature, can impact the relationship between skin blood flow and core temperature by altering the threshold and/or sensitivity of the responses.

For example, a lower skin temperature, possibly due to greater evaporative cooling at altitude, will result in a shift in threshold for active vasodilation and sweating to a higher core temperature and a decreased slope of the skin blood flow/sweat rate-to-core temperature relationships. Along these lines, Wenger reported that a 1°C change in skin temperature is sufficient to reduce the slope of the skin blood flow-to-core temperature

relationship by 12-13% (50). In contrast, exercise and dehydration will independently increase the core temperature threshold for vasodilation and sweating. Other factors, such as changes in blood osmolality, blood volume, and posture can also influence thermoregulatory responses. In general, any influence viewed by the thermoregulatory control centers as an additional thermal challenge will typically lower thresholds, and any challenge to blood pressure regulation will result in higher thresholds.

In order to investigate the effects of hypoxia on non-acral skin blood flow in thermoneutral conditions, we recently measured cutaneous vascular responses in non-acral skin using laser-Doppler flowmetry under seven separate conditions in healthy men and women. We compared the skin blood flow response (as cutaneous vascular conductance) during 1) spontaneous breathing, 2) controlled breathing matching their individual spontaneous rate and depth, 3) increased tidal volume, 4) increased respiratory rate, 5) isocapnic hypoxia (arterial saturation ~85%), 6) controlled breathing to match ventilation during hypoxia, and 7) controlled breathing to match hypoxic breathing frequency. Although the responses to hypoxia were somewhat variable, we observed increased skin blood flow during isocapnic hypoxia and determined that these responses were not due to increased breathing rate or tidal volume. Although we did not evaluate the effects of hypocapnia in this preliminary study, hypocapnia accompanying hypoxic hyperventilation may contribute to thermoregulatory impairment and a lowering of core body temperature (12, 13).

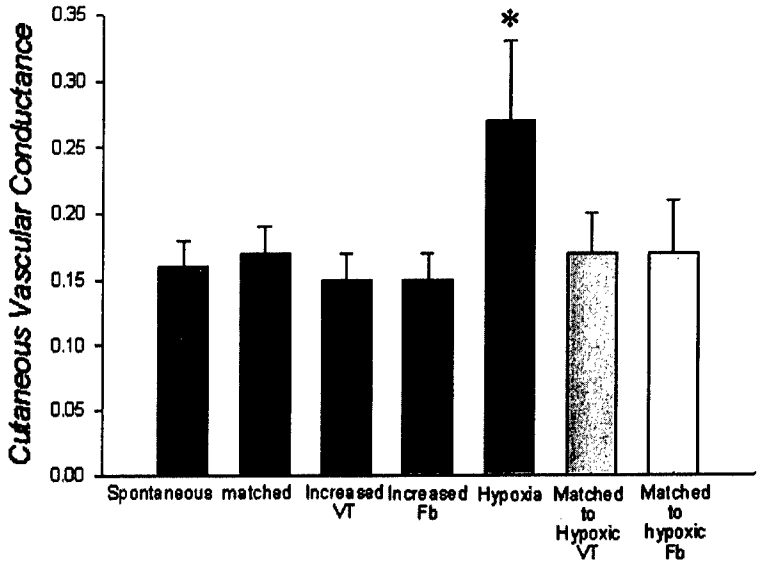

Figure 3. Non-acral skin blood flow responses to hypoxia in thermoneutral conditions. Skin blood flow (as cutaneous vascular conductance) was measured during 1) spontaneous breathing, 2) controlled breathing matching their individual spontaneous rate and depth, 3) increased tidal volume (VT), 4) increased respiratory rate (F_b), 5) isocapnic hypoxia (arterial saturation ~85%), 6) controlled breathing to match ventilation during hypoxia, and 7) controlled breathing to match hypoxic breathing frequency. Hypoxia increased skin blood flow independent of any changes in respiration.

Our findings in non-acral skin agreed with those of Sagawa *et al.* (39), in which they found a doubling of forearm blood flow (measured by venous occlusion plethysmography) at a simulated altitude of 5,600m. Similarly, studies at altitude have demonstrated elevated skin temperatures at a given ambient temperature, suggesting peripheral vasoconstriction may be reduced by hypoxia (1, 2).

Importantly, most studies investigating the effects of hypoxia on non-acral skin blood flow have used venous occlusion plethysmography or skin temperature as indexes of skin blood flow. Although skin temperature is a function of underlying blood flow, it is also affected by environmental conditions and evaporation. One problem with venous occlusion plethysmography is that changes in blood flow in both skin and the underlying muscle are measured. Thus, conclusions drawn about skin blood flow using this technique assume that blood flow to muscle has not changed. It is now clear that hypoxia can have profound effects on muscle blood flow, potentially obscuring or exacerbating true changes in skin blood flow when using this technique (see accompanying reviews, (7, 16)). In contrast, laser-Doppler flowmetry allows one to measure changes in skin blood flow without measuring concomitant changes in the underlying muscle. However, laser-Doppler flowmetry is not without limitations, and investigators must use caution when designing studies using this technique.

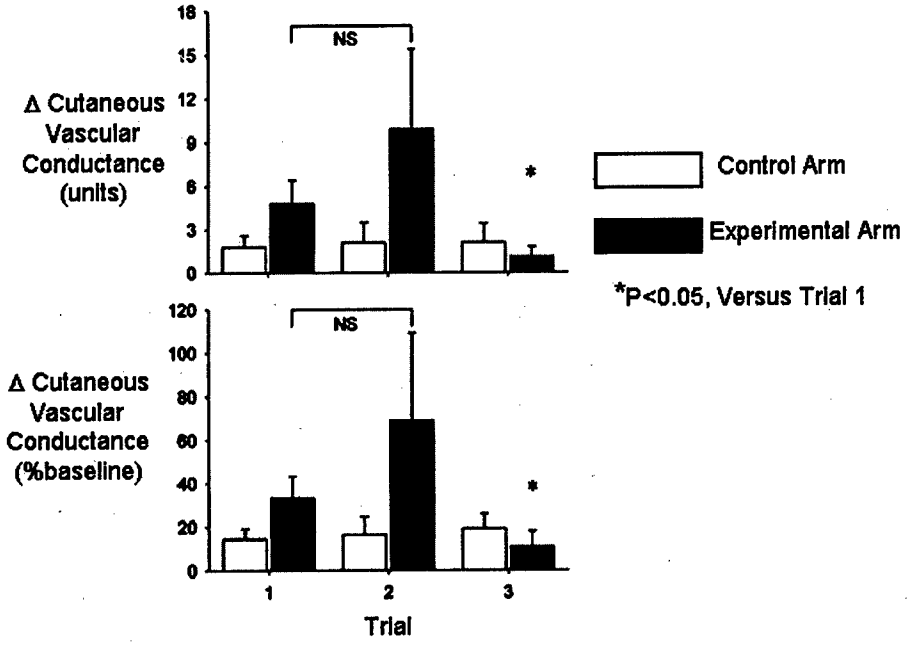

Figure 4. Non-acral skin blood flow responses to hypoxia. The change (upper panel) and percent change (lower panel) in cutaneous vascular conductance in the control arm and experimental arm are shown for the three hypoxia trials. In Trial 1, the experimental arm received the α-receptor blocker phentolamine. In Trial 2, the experimental arm received phentolamine and the β-receptor blocker propranolol. In Trial 3, the experimental arm received phentolamine, propranolol, and L-NMMA to inhibit nitric oxide production. Data from Reference (49).

To further investigate the mechanisms of hypoxic vasodilation in non-acral skin, we measured cutaneous vascular conductance using laser-Doppler flowmetry in forearm skin to isocapnic hypoxia after selective α-adrenergic blockade with phentolamine (49). We found that in the absence of α-adrenergic blockade greater cutaneous vasodilation was unmasked, similar to our finding in skeletal muscle (49). In contrast to our findings in skeletal muscle, subsequent β-receptor blockade with phentolamine did not reduce cutaneous vasodilation despite the existence of functional β-receptors in non-acral skin (3). Infusion of L-NMMA, on the other hand, significantly reduced cutaneous vasodilation during isocapnic hypoxia to values below control sites not receiving any drug infusions. At present, we are unable to ascertain whether hypoxic vasodilation in the skin during normothermia is mediated by NO or is merely NO dependent.

REGULATION OF NON-ACRAL SKIN BLOOD FLOW DURING EXERCISE

The vast array of factors that can influence the core temperature-to-skin blood flow/sweating relationships by thermoregulatory control centers makes designing studies investigating interactions between hypoxic and thermoregulatory reflexes extremely challenging. For example, the initial contraction of plasma volume at altitude may impact the thermoregulatory reflexes by increasing the threshold for vasodilation and sweating as discussed above. Thus, at a given core temperature, the absolute level of skin blood flow or sweat rate will be reduced. Although this may be interpreted as an effect of hypoxia on cutaneous vascular tone, this is not really correct as the same change in plasma volume at sea level may similarly change the thresholds, assuming all else was the same in the two conditions. These centrally-mediated changes in thermoregulation may obscure any effects of hypoxia on cutaneous vascular tone at the level of the cutaneous smooth muscle.

Exercise is another factor that can modify the core temperature-thermoregulatory response relationships in a very complex manner, particularly when exercise is performed in a hypoxic environment. If exercise could be performed adiabatically, that is, if none of the heat produced were lost to the environment, core temperature would rise linearly and continuously throughout the duration of the exercise bout. In this situation, the rate of rise of core temperature would depend entirely on the rate of metabolic heat production minus any external work performed. However, steady-state internal temperature during exercise at a constant intensity is dependent on several factors. For a given individual, metabolic heat production may be dependent on absolute intensity, but steady-state core temperature correlates better with relative intensity (i.e., $\%VO_{2max}$) than with absolute oxygen consumption (40). The important point here is that heat loss mechanisms, and therefore core temperature, are affected by exercise according to the relative exercise intensity. Thus, when exercise in a hypoxic environment is compared to the same absolute workload at sea level, the absolute heat production may be similar in the two environments, but heat loss mechanisms may be activated to a lesser degree in the hypoxic environment, as the relative workload is greater owing to the reduction in maximum O_2 uptake.

In an attempt to overcome some of the challenges to understanding the effects of a hypobaric environment on regulation of skin blood flow and sweating during exercise, Kolka and colleagues (22) controlled the work intensity at each altitude studied so that there

was a consistent thermoregulatory perturbation. These authors used a relative exercise intensity of 60% altitude-specific VO_{2peak} as a "thermal clamp" to limit the change in core temperature, and measured the thresholds and sensitivities (slopes) for vasodilation and sweating at 770 torr (sea level), 596 Torr (2,596 m), and 462 Torr (4,575m). They observed no changes in threshold for sweating, but a decreased slope of the mean sweat rate-to-core temperature relationship at each altitude. Furthermore, they also observed a significantly decreased slope of skin blood flow rise in most subjects at the highest altitude. Importantly, evaporation of sweat is greater in a hypobaric environment, as maximal evaporative capacity is enhanced due to an increase in effective mass transfer coefficient for any given air movement (29).

In the study by Kolka *et al.* (22), greater evaporation of sweat at altitude caused lower skin temperatures in the hypoxic environments at a given core temperature. As discussed above, a decreased local skin temperature will suppress the sensitivity of cutaneous vasodilation. In this context, hypoxemia may not have directly impacted skin blood flow, but the effect of hypobaria on sweating, and therefore skin temperature, may have resulted in an attenuated skin blood flow response to an increased core temperature.

In contrast to the findings of Kolka *et al.*, Greenleaf and colleagues showed an increase in whole-body evaporative heat loss at altitude, but attributed the difference entirely to greater respiratory water loss (15). These authors did not observe differences in steady-state sweating rate at altitude, even though subjects worked a greater intensity at altitude (65% of altitude VO_{2max}) than at sea level (45% of sea-level VO_{2max}). In this study, the authors did not evaluate the sensitivity of the responses as core body temperature increased during exercise.

Others have reported that hypoxia may result in a lowered oxygen tension at the sweat gland and affect synthesis of acetylcholine, thus reducing synaptic transmission. Elizondo (9) observed decreased sweat rates during arterial occlusion. However, the decreased sweat rates were reversed when physostigmine, an anticholinesterase, was administered in combination with arterial occlusion. Recently, DePasquale and colleagues observed decreased sweat rate to pilocarpine iontophoresis in subjects exposed to normobaric hypoxia simulating an altitude of 3050m ($O_2\%$ = 13.9; (6)). Taken together, these studies suggest that hypoxia may alter sweat rate at the level of the sweat gland by interfering with neural transmission of acetylcholine.

Sagawa and colleagues (39) did not observe an attenuated skin blood flow response in non-acral skin during heat stress at a simulated altitude of 5,600m. Similarly, Rowell and colleagues did not show any alteration in non-acral skin blood flow during exercise in a hypoxic breathing study (37). These findings were surprising in light of Rowell's previous observations that the splanchnic region does not demonstrate the typical vasoconstriction observed during exercise, when exercise is performed in a hypoxic environment (35). As both the cutaneous and splanchnic vascular beds are very compliant, it seems unlikely that both circulations can receive a high blood flow during exercise in the heat without significantly reducing cardiac filling pressure, stroke volume, and cardiac output. Importantly, both of these studies used venous occlusion plethysmography to measure forearm blood flow and extended their observations to the skin. Blood flow to resting skeletal muscle will decrease during whole body heating (34), and it seems reasonable to assume that this would occur to a greater extent during combined stresses of exercise, heat stress, and hypoxia. Thus, either changes in skin blood flow have not been consistently observed dur-

ing hypoxic conditions due to limitations in measurement methods, as discussed above, or the lack of splanchnic vasoconstriction observed during hypoxic exercise does not occur to the same extent when hypoxic exercise is performed in the heat. It is clear that more research is warranted to address these issues.

It is important to note that there have been no studies in which blood flow in non-acral skin has been measured during heat stress after prolonged exposure to a hypoxic environment (in this case, more than a few hours). It is likely that additional factors, such as the contracted plasma volume known to occur with more prolonged exposure to a hypoxic environment, may have profound influences on skin blood flow and sweating.

HYPOXIA AND REGULATION OF CORE BODY TEMPERATURE

Basal metabolic rate, and therefore total heat production, is increased with altitude exposure. However, hypoxia lowers core temperature during hypoxic exposure in animals (11, 14), and in humans breathing hypoxic gas mixtures at thermoneutral and cool temperatures (32) and at altitude in the cold (2). As most of the body surface area of humans is covered with non-acral skin, these findings are not surprising, and suggest a greater core cooling rate may occur with hypoxia. A recent study by Johnston and colleagues (19) addressed this issue by studying the core cooling rate in humans following a bout of exercise in 28°C water while breathing an isocapnic hypoxic gas mixture. They found that isocapnic hypoxia lowered core temperature thresholds for vasoconstriction and shivering, and increased core cooling rate by 33%. They attributed their findings to a delay in the onset of vasoconstriction and shivering as well as increased respiratory heat loss during hypoxic hyperventilation.

HYPOXIA AND REGULATION OF ACRAL SKIN BLOOD FLOW

In contrast to non-acral skin, it is generally agreed that acral skin lacks influence from active vasodilator nerves. Therefore, reflex control of skin blood flow in these regions is thought to be controlled entirely by the noradrenergic vasoconstrictor system (18). Skin blood flow in acral skin is characterized by large spontaneous fluctuations, due to changes in blood flow through arteriovenous anastamoses (AVA's). It is estimated that the magnitude of total flow fluctuations in the hands and feet is approximately 5-10% of cardiac output in resting thermoneutral conditions. In a resting, thermoneutral condition, AVA's constrict two or three times a minute, and have been shown to have a significant relationship to blood pressure and heart rate variability. Vasomotion is believed to be synchronous in all skin AVA's, as blood flow variations in arteries supplying separate areas of skin, such as the hand or the foot, are found to be highly correlated (26, 46).

Blood flow fluctuations in acral skin are most pronounced in a thermoneutral environment, and contribute to maintaining a stable core temperature. A number of studies have clearly demonstrated a relative vasoconstriction in the hand to hypoxic conditions (8, 10, 48, 52) as the result of increased sympathetic outflow (23). It is unknown whether blocking α-receptors would unmask hypoxic vasodilation in acral skin, similar to that observed in non-acral skin, as this has not been done.

Passino and colleagues (30) reported that acral skin blood flow in the fingers showed reduced vasomotor variability in high-altitude residents (an index of vasoconstriction in thermoneutral conditions) compared to sea level residents. Furthermore, they reported a much greater vasoconstriction in acclimatized lowlanders (after 1 week at altitude) than in high altitude residents. Taken together, these findings suggest that acral skin strongly vasoconstricts in response to high altitude, and that this vasoconstriction is still observed after chronic exposure to a hypoxic environment.

In situations where there is a need for heat conservation, the AVA's are mainly closed, whereas if there is a need for heat elimination, the AVA's are mainly open, resulting in greatly reduced fluctuations at high and low body temperatures. However, Sagawa and colleagues (39) found blood flow to be reduced in the fingers during a 60 minute heat stress at a simulated altitude of 5,600 meters compared to sea level conditions. This decreased acral skin blood flow may have contributed to the faster rise in core temperature to heating while at altitude in this study.

Local skin temperature also greatly affects blood flow in acral skin. When a human finger is immersed in water between 15 and 21°C, the skin vasoconstricts; however, when immersed in water less than 15°C, skin temperature falls and remains low for 5-10 minutes, but then abruptly increases. This abrupt rise in skin blood flow and skin temperature is termed the "hunting reaction" first described by Lewis (25) or, more recently, "cold-induced vasodilation". This reflex is thought to be a protective mechanism to minimize the risk of cold-induced damage to the skin. The increase in blood flow is caused by dilation of the AVA's but the exact mechanism(s) is poorly understood. It has been suggested that the high blood flow is caused by cold paralysis of the smooth muscle cells (45). Interestingly, individuals who chronically expose their hands to cold water show enhanced cold-induced vasodilation responses (28).

Cold-induced vasodilation is significantly reduced at high altitudes (27, 31), and may increase the risk for frostbite injuries. The cold-induced vasodilation response does not return to normal during short-term acclimatization, but rapidly returns to normal upon return to sea level (5). This may suggest that cold-induced vasodilation is affected by the tissue or blood hypoxemia or hypocapnia associated with high altitude exposure. Alternatively, augmented levels of chronic vasoconstriction in acral skin at altitude may contribute to diminished cold-induced vasodilation by favoring the balance of vasoconstrictors to vasodilators. However, Purkayastha (31) recently reported that vitamin C supplementation at altitude (3,700m) improved cold-induced vasodilation responses in the hand. They suggested that degenerative changes in mitochondrial function by oxygen free radicals generated under hypoxic stress may contribute to the decreased blood flow to the periphery, and that antioxidants may help protect against cold-induced injuries.

SUMMARY

There have been relatively few studies investigating the potential effects of hypoxia on the regulation of cutaneous vascular tone, particularly in non-acral skin. Of the studies that have been done, there seems to be a high degree of variability in the responses observed. Part of the discordant observations stem from the fact that responses to both hypoxia and thermal challenges involve complex neural and local vascular changes. Furthermore, a

number of studies have used techniques that do not directly measure skin blood flow, making conclusions drawn from these studies tenuous, at best. Despite these challenges, cutaneous vascular responses to hypoxia can presently be summarized as follows. In thermoneutral conditions, non-acral skin appears to vasodilate during hypoxia, whereas acral skin vasoconstricts. Hypoxia also appears to affect thermoregulatory responses. Hypoxia reduces the slope of the skin blood flow/sweat rate-to-core temperature relationships in a hot environment, but appears to have no effect on the threshold for activation of sweating. Hypoxia reduces cold-induced vasodilation in acral skin, and enhances core cooling rate by delaying the onset of vasoconstriction and shivering in a cold environment. More studies are needed to elucidate the mechanisms that underlie these changes in regulation of cutaneous vascular tone in humans.

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Chapter 19

TURNING UP THE HEAT IN THE LUNGS
A key mechanism to preserve their function

Claudio Sartori and Urs Scherrer

Abstract: Life threatening events cause important alterations in the structure of proteins creating the urgent need of repair to preserve function and ensure survival of the cell. In eukariotic cells, an intrinsic mechanism allows them to defend against external stress. Heat shock proteins are a group of highly preserved molecular chaperones, playing a crucial role in maintaining proper protein assembly, transport and function. Stress-induced upregulation of heat shock proteins provides a unique defense system to ensure survival and function of the cell in many organ systems during conditions such as high temperature, ischemia, hypoxia, inflammation, and exposure to endotoxin or reactive oxygen species. Induction of this cellular defense mechanism prior to imposing one of these noxious insults, allows the cell/organ to withstand a subsequent insult that would otherwise be lethal, a phenomenon referred to as "thermo-tolerance" or "preconditioning". In the lung, stress-induced heat shock protein synthesis, in addition to its cyto-protective and anti-inflammatory effect, helps to preserve vectorial ion transport and alveolar fluid clearance. In this review, we describe the function of heat shock proteins in the lung, with particular emphasis on their role in the pathophysiology of experimental pulmonary edema, and their potential beneficial effects in the prevention and/or treatment of this life-threatening disease in humans.

Key Words : heat shock proteins, lung, acute respiratory distress syndrome, alveolar fluid clearance, epithelial sodium channel

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STRESS-INDUCED PROTEIN DENATURATION INCREASES THE EXPRESSION OF HSP

In 1962 Ritossa observed that exposing *Drosophila* to elevations of temperature produced "puffing" patterns of polytene chromosomes indicating increased gene activity (18). Approximately 10 years later, Tissières and colleagues demonstrated that these "puffing" patterns represented upregulation of genes encoding for heat shock proteins (HSP) (26). This heat shock response, now commonly referred to as the stress response, is ubiquitous in nature and consists of the transcription and translation of a set of HSPs, which possess a tremendous homology across virtually all living cells.

HSPs are proteins ranging from 8–110 kDa that are assigned to families on the basis of sequence homology and typical molecular weight (33, 34). In eukaryotes, there exist many families that comprise multiple members, differing in degree and kinetics of inducibility, intracellular distribution, tissue specificity and function (3, 4).

Table 1. Heat shock protein families, localization and function

NAME	kDA	LOCALISATION	FUNCTION
Ubiquitin	8	Cytosol/nucleus	Degradation
HSP 27	27	Cytosol/nucleus	Molecular chaperone; cytoprotection
Heme Oxygenase	32	ER and cytoplasm	Resistance to oxidant stress
HSP 47	47	ER	Collagen chaperone
HSP 60	60	Mitochondria	Molecular chaperone
HSP 70	72	Cytosol/nucleus	Cytoprotection
HSP 90	90	Cytosol/nucleus	Regulation steroid receptor activity
HSP 110	110	Nucleolus/cytosol	Nucleoli protection from stress

MECHANISMS CAUSING INDUCTION OF HSP EXPRESSION

In addition to elevated temperatures, induction of HSP expression has also been observed under various other conditions such as ischemia, oxygen deprivation, inflammation, or exposure to endotoxin, reactive oxygen species, ethanol, heavy metals or other chemical denaturants. All these different forms of stress may induce protein conformational changes either directly or indirectly.

Accumulation of denatured or abnormally folded proteins itself is assumed to represent the key proximal signal for initiation of the stress response in a given cell or tissue (27). The exact underlying mechanisms by which denatured proteins initiate the stress response

are incompletely understood, but are thought to relate to the ability of denatured proteins in the cytoplasm to stimulate a cascade of interactions between heat shock protein and a series of co-chaperones such as heat shock factors (HSF) and heat shock elements (HSE) which finally results in activation of the HSP promoter and a dramatic and rapid increase in specific stress protein expression (4).

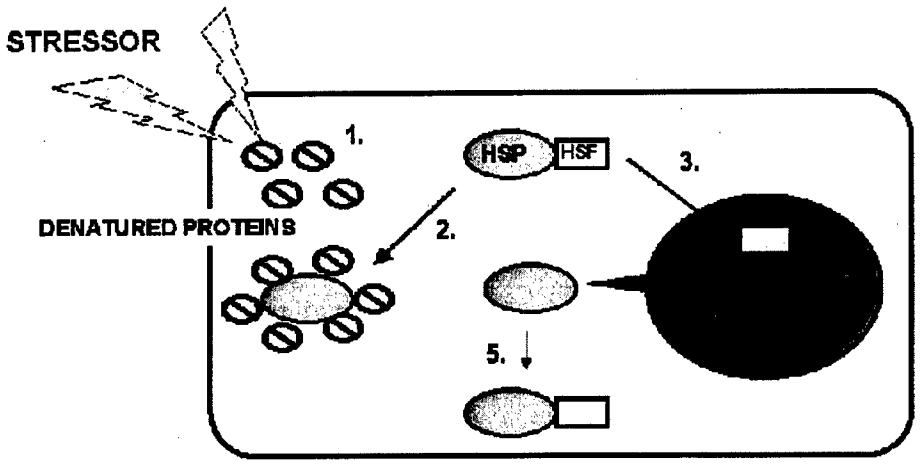

Figure 1. During stress, denatured proteins (1) are bound by existing intracellular pools of HSP70 (2), causing a relative depletion of unbound HSP70. The decreased level of intracellular HSP70 shifts the equilibrium between HSF and HSP70, thus liberating HSF to trimerize, translocate to the nucleus (3), and activate HSP70 transcription (4) via high-affinity binding with the HSE (heat shock elements). When the level of newly synthetized HSP70 reaches some critical level, the equilibrium between HSF and HSP70 is restored (5), and HSF activation is terminated. HSF can then translocate to the nucleus and interact with heat shock elements in the promoters of *HSP70* and other target genes.

Increased HSP mRNA transcripts are present already a few minutes after a stress occurs, whereas protein accumulation reaches its maximum roughly 12 hours after stress induction. Thereafter, HSP content in tissues slowly decreases, but may remain elevated up to 192 hours after the initial stimulus.

CYTOPROTECTIVE EFFECTS OF INCREASED HSP EXPRESSION

Although the precise function of the stress proteins is not known, it is clear from a number of studies that they have cytoprotective effects. Heating cells to a few degrees Celsius above their resting temperature for a short period of time confers protection a few hours later to a second heat stimulus that would otherwise be lethal: a phenomenon described as thermo-tolerance. Furthermore, heating also confers tolerance to other, non-thermal, noxious stimuli, and conversely, induction of the stress response by non-thermal means

can induce thermo-tolerance. The term cross-tolerance has been coined to describe this phenomenon (27, 40).

The mechanisms by which the stress response and stress proteins confer cytoprotection are still poorly understood. As molecular chaperones, stress proteins are known to transiently stabilize and refold damaged intracellular proteins and prevent intracellular protein aggregation during stress. Alternatively, several other protective functions have been attributed to HSPs.

An important feature of the stress response is that increased HSP expression is associated with a concomitant transient shut-down of non-stress protein gene expression. Based on this observation, it has been postulated that stress response-mediated inhibition of gene expression, particularly pro-inflammatory gene expression, may be one of the mechanisms by which the stress response protects against acute injury.

Protective effects of HSPs have also been attributed to their ability to 1) decrease the intracellular level of radical oxygen species (ROS, and, in turn, modulate glutathione metabolism to maintain it in reduced state 2) suppress apoptotic signaling pathways (inhibition of JNK-mediated apoptosis, inhibition of caspase activity); and (3) interact with nitric oxide-induced cytoprotection (4).

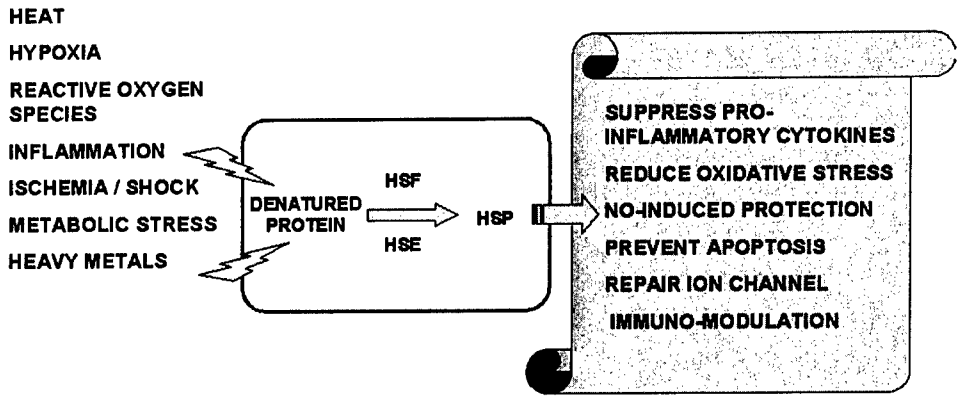

Figure 2. Proteotoxic stressors and cytoprotective effects of heat shock proteins

In summary, the stress response is a highly conserved evolutionary adaptation designed to quickly remove damaged proteins and restore the normal protein folding environment of cells following a proteotoxic insult. Even if not exclusively, this protection is largely attributable to induction of specific heat shock protein expression. Based on this concept, novel therapeutic strategies using pharmacologic interventions and/or gene transfection techniques are being investigated for their potential to enhance HSP expression by the cells. In cardiovascular disease such strategies have been intensively investigated to improve the tolerance of myocardial cells against ischemic insults and, thereby, improve the outcome and survival of patients suffering from ischemic events.

HSP IN THE LUNGS

That the stress response may also play a critical role in lung biology is easily predictable given its highly conserved nature. Surprisingly, however, its role has begun to be elucidated only very recently. Among the many classes of stress proteins, heme-oxygenase-1 (HO-1) and heat shock protein 70 are the best characterized with respect to lung biology (40). Hypoxia is a potent, but transient inducer of HO-1 in vascular smooth muscle cells *in vitro* and in the lung *in vivo* (6).

Stress protein expression has been well described in whole lungs and in specific lung cells from various species. Cultured pulmonary artery endothelial cells, airway epithelial cells, pulmonary artery smooth muscle cells and alveolar macrophages express abundant HSP70 after thermal stress (40). In patients suffering from cancer, asthma, or acute lung injury, augmented HSP expression has been reported in the lung *in vivo*.

STRESS PROTEINS HAVE AN IMPORTANT CYTOPROTECTIVE ROLE DURING LUNG INFLAMMATION AND INJURY

Five years after Ritossa's description of the heat-induced puffing patterns of polytene chromosomes in the Drosophila, Ashbaugh and colleagues described a new clinical syndrome that they called the acute respiratory distress syndrome (ARDS) (1).

ARDS is a form of non-cardiogenic pulmonary edema, associated with pulmonary infiltrates, stiff lungs, and severe hypoxemia which affects 50-75 per 100,000 population per year and leads to the demise of 30–50% of affected patients, principally because of sepsis or multiple organ dysfunction.

ARDS is an inflammatory disease characterized by an imbalance between pro- and anti-inflammatory compounds such as cytokines, and abnormalities of the coagulation system. Its pathology comprises hyaline membranes, endothelial and epithelial injury, loss of epithelial integrity, and increased alveolar-capillary permeability resulting in diffuse alveolar damage, with neutrophils, macrophages, erythrocytes, hyaline membranes, and protein-rich edema fluid in the alveolar spaces.

This loss of alveolo-capillary integrity, increases fluid flux into the alveoli, and thereby causes the clinical manifestations of ARDS. In addition, alveolo-capillary barrier leakiness can also lead to loss of lung compartmentalization, with the result that inflammatory mediators from the lung can enter the circulation and induce systemic consequences (multiple organ dysfunction).

After the acute phase of acute lung injury and the acute respiratory distress syndrome, some patients have an uncomplicated course with rapid resolution of the disorder. Other patients show progression to fibrotic lung injury which can be observed histologically as early as five to seven days after the onset of the disorder. The alveolar space becomes filled with mesenchymal cells and their products, along with new blood vessels.

The underlying mechanisms leading either to resolution of the inflammatory-cell infiltrate or fibrosis are unclear. Apoptosis (programmed cell death) is thought to be a major mechanism for the clearance of neutrophils from sites of inflammation and may be important for the clearance of neutrophils from the injured lung (31).

ROLE OF HEAT SHOCK PROTEINS AS POTENTIAL PHYSIOPATHOLOGICAL ACTORS AND THERAPEUTIC TARGETS

The treatment of ARDS is merely supportive because the patho-physiology of this highly lethal disease is poorly understood. Recognition of some key components of ARDS such as inflammation, epithelial dysfunction, apoptosis and fibrosis, prompted interest in the role of heat shock proteins as potential physiopathological actors and possible therapeutic targets. The evidence is as follows:

During acute lung injury several non-thermal inducers of stress proteins such as oxidant injury, inflammation, and ischemia-reperfusion are present. Moreover, recent data show that HSP-70 can limit the inflammatory response, protect proteins from damage, restore function of proteins that are damaged, and prevent cell destruction in lung tissues. Several examples of stress protein-mediated cytoprotection exist in cell and animal models of acute lung injury (23, 32, 40).

IN VITRO STUDIES

In vitro studies indicate that several mechanisms may account for the favorable effects of HSP in the lung. Recent *in vitro* studies have demonstrated that in pulmonary cells, cytoprotective effects of HSP may involve attenuation of endotoxin-mediated apoptosis and/or antioxidant effects (35).

Alternatively, by binding to cytokines and preventing their release from inflammatory cells, HSPs also have anti-inflammatory effects. In the cultured human respiratory epithelium, induction of the stress response inhibited tumor necrosis factor-alpha and prointerleukin-1B gene expression (40).

HSP70 binds intracellular tumor necrosis factor-alpha and prevents its release from the cells, an effect that has been suggested to be mediated by NF-kB. Indeed, HSP70 overexpression by plasmid-mediated gene transfer inhibits nuclear factor-kB (NF-kB) nuclear translocation (39).

Another important aspect of the stress response-mediated protection by HSP is related to inhibition of iNOS gene expression. In cultured rat pulmonary artery smooth muscle cells and murine respiratory epithelium, the stress response inhibits cytokine-mediated iNOS gene expression without affecting cell viability (37, 38).

Interestingly in cultured pulmonary cells the stress-induced suppression of proinflammatory gene expression appears to be selective, not generalized because surfactant protein expression is preserved (36).

IN VIVO STUDIES

Consistent with these positive results *in vitro*, studies *in vivo* and *ex-vivo* animal models have shown protective effects of HSP in experimental acute lung injury. Villar *et al.* were the first to demonstrate a cytoprotective effect of stress protein induction in a rat model of

acute lung injury caused by intratracheal administration of phospholipase A1. HSP70 was induced in the lungs of experimental animals by subjecting them to whole body hyperthermia (41C for 15 minutes) 18 hours before phospholipase administration. Heat-treated animals were significantly resistant to phospholipase A1-mediated acute lung injury, and had decreased mortality at 48 hours compared with control (non-heated) animals (28). Using the same heat preconditioning model, it was subsequently demonstrated that stress protein induction also protected against lung injury caused by intratracheal administration of TNF-alpha or systemic administration of endotoxin (30). More importantly, the whole body heating-induced stress response also had protective effects against acute lung injury when initiated after an endotoxin challenge (17). In these models, increased survival was correlated with blunted endotoxin-mediated iNOS mRNA expression in the lung, significant reduction of peak plasma concentration of cytokines (in particular IL-1-beta), attenuated neutrophil recruitment (11), and decreased microvascular protein permeability (5).

Similar positive results were obtained in another experimental model of lung injury: the ventilator-induced acute lung injury. Following mechanical ventilation with high tidal volume, heat preconditioned lungs had smaller decrease in lung compliance, lower plasma cytokine levels (TNF-alpha, Interleuline-1 beta, macrophage inflammatory protein-2) and an increased amount of active surfactant aggregate in BAL, compared to lungs from non-preconditioned animals (16, 29).

Taken together, although the mechanism of protection or the involvement of specific stress proteins remain incompletely understood, these studies suggest that stress protein induction could represent a novel therapeutic strategy for acute lung injury. However, an important limitation of these studies was that the stress response was produced by heating the animals or by using sodium arsenite, approaches that are not readily amenable to clinical application. Moreover, these treatments caused a full systemic stress response, and did therefore not reveal the underlying mechanism of protection in a given organ system.

To overcome some of these limitations, Weiss and colleagues tested the hypothesis that direct intratracheal adenoviral-mediated overexpression of the HSP-70 would improve the outcome of acute lung injury secondary to cecal ligation and perforation (a standard model for producing sepsis and a subsequent ARDS-like syndrome) in mice *in vivo*. The results were impressive: 48 hour mortality was cut in half, and edema and neutrophil accumulation in the alveolar space of treated mice were significantly attenuated (32). Consistent with these observations, adenovirus-mediated transfer of the stress protein heme oxygenase-1 cDNA into the lungs attenuates the severity of lung injury induced by the influenza virus in mice (9).

In summary, these data in experimental animals suggest that the stress response has selective inhibitory effects on the expression of genes relevant to lung injury and function. The mechanisms by which the stress response protects against ALI may involve selective inhibition of potentially deleterious patterns of gene expression (i.e. iNOS, TNF-a, and other NF-kB-mediated inflammatory processes) while allowing ongoing expression of beneficial patterns of gene expression (i.e. surfactant protein). Finally, selective adenovirus-mediated overexpression of stress proteins in the mouse augments survival after acute lung injury.

HEAT SHOCK PROTEINS, CHAPERONES AND RESPIRATORY TRANSEPITHELIAL ION TRANSPORT IN ACUTE LUNG INJURY

Because epithelial injury contributes to pulmonary edema by facilitating alveolar flooding and disrupting normal transepithelial ion and alveolar fluid clearance mechanisms, the degree of alveolar epithelial injury is an important predictor of the outcome of ARDS. Strategies that hasten the resolution of pulmonary edema may therefore be as important as those that attenuate early inflammatory lung injury, as suggested by the observation showing that maintenance of the ability to remove alveolar fluid is associated with improved oxygenation, a shorter duration of mechanical ventilation, and an increased likelihood of survival (12, 31).

Pulmonary edema results from a persistent imbalance between forces driving fluid into the airspaces and biological mechanisms for its removal. There is abundant evidence that active ion transport across the alveolar epithelium creates an osmotic gradient that leads to alveolar fluid clearance both during the perinatal period and in the adult lung. Sodium enters the apical membranes of alveolar epithelial cells through amiloride-sensitive cation channels, such as the epithelial sodium channels (ENaC) and the non-selective cation channels, and is then transported across the basolateral membrane into the interstitium by the ouabain-inhibitable Na-K-ATPase. ENaC is thought to be the limiting factor regulating transepithelial sodium transport and alveolar fluid clearance in the lung, because even a small fraction of the normal Na-K-ATPase activity appears to be sufficient to maintain normal ion transport (22).

In humans, indirect evidence suggests that a possibly genetic and /or acquired (see next paragraph) impairment of transepithelial sodium and water transport predisposes to high-altitude pulmonary edema (HAPE) (21), and plays a role in the pathogenesis of the RDS of the newborn (2).

Recently, increased transporter movement from putative intracytoplasmic pools to the cell membrane (intracellular trafficking) and stability of the transporter at the cell membrane has been suggested to stimulate ion transport (19, 25), but in particular with regard to ENaC this possibility is not proved. A defect in protein processing of membrane transporters has been shown to play a role in human disease such as cystic fibrosis (inefficient folding of the chloride channel CFTR) and Liddle's syndrome (increased stability of the ENaC at the cell membrane) (19).

In the kidney only a few percent (1-5%) of the ENaC synthesized in the endoplasmic reticulum reaches the cell surface (Figure 3). This may be due to rapid destruction of ENaC, related to incomplete protein folding and rapid channel degradation by endocytosis and ubiquitination (19).

It is well established that specific disease-related factors (for example: hypoxia/ hypoxemia, nitric oxide, cytokines, reactive oxygen species or pro-apoptotic molecules) downregulate sodium and water transport across the alveolar epithelium, and thereby impair alveolar fluid removal and favor pulmonary edema (22). The underlying mechanisms are still poorly understood, but, as recently shown for hypoxia, may involve dysregulation of ENaC processing and stability to the membrane (15) (Figure 3).

These observations could be consistent with the hypothesis that a genetic and/or ac-

quired defect of ENaC processing in the lung may augment the susceptibility to pulmonary edema, whereas increased efficiency of this processing may prevent alveolar fluid flooding during lung injury. This has led to studies examining the effects of interventions aimed to augment protein processing, such as stress-preconditioning or chemical chaperones, on respiratory transepithelial ion and water transport.

Upregulation of the heat shock protein 70 has been shown to stimulate intracellular processing of the chloride channel CFTR and partially restore its function in cells with a genetic defect of the intracellular processing of this channel (which has been shown to interact with the ENaC to regulate the respiratory transepithelial ion transport) (7). More importantly, administration of chemical compounds having chaperone activities similar to those characteristic for heat shock proteins, increased CFTR membrane expression and transepithelial chloride transport not only in mice with a genetic defect of CFTR processing, but also in their wild-type littermates (8) (Figure 4). Finally, during ischemia/ reperfusion-induced lung injury, stress proteins allow to restore the ion and fluid transport capacity of the alveolar epithelium by upregulating alveolar fluid clearance in response to catecholamines (14) (Figure 5).

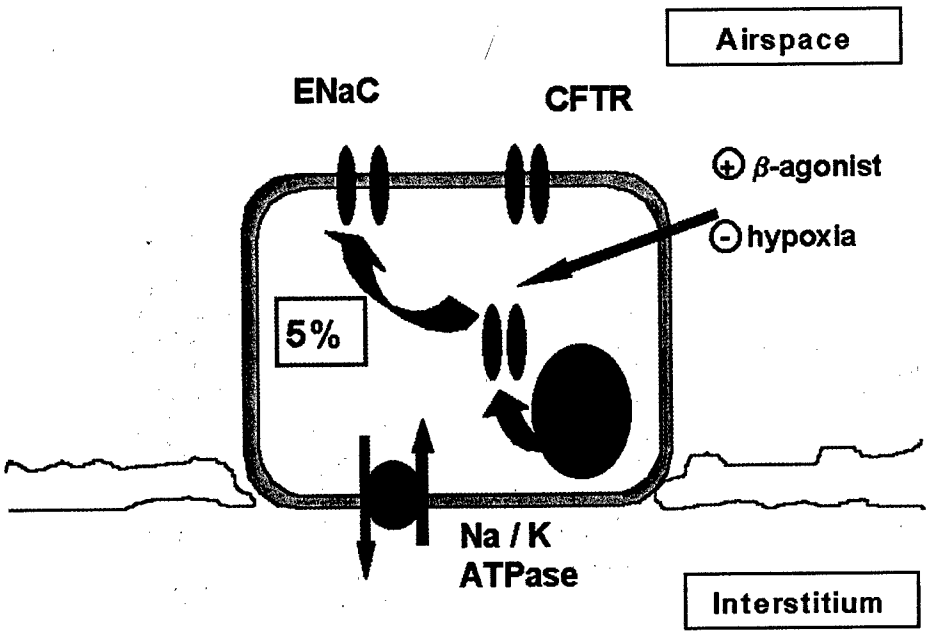

Figure 3. Only a few percent (1-5%) of the ENaC synthesized in the endoplasmic reticulum reaches the cell surface. It was recently suggested that the intracellular processing of the ENaC may be modulated by external factors such as drugs or disease-related factors. Whether endogenous (HSPs) or exogenous (chemical chaperones) may also influence apical ion channels processing, and in turn transepithelial sodium transport in alveolar type II cells is currently under investigation.

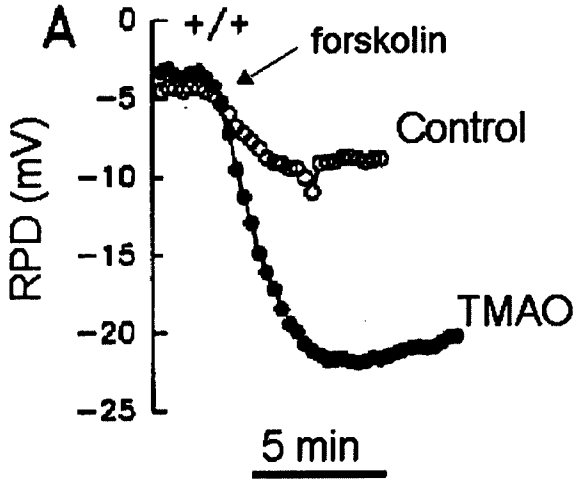

Figure 4. Effect of the chemical chapterone TMAO on chloride transport in the rectum of mice. The chemical chaperone trimethyl amino oxide (TMAO) increases the forskolin-dependent rectal potential difference (RPD) in wild type mice. This suggests that chemical chaperones may represent a novel therapy to augment ion channels expression at the cell membrane and transepithelial ion transport (8).

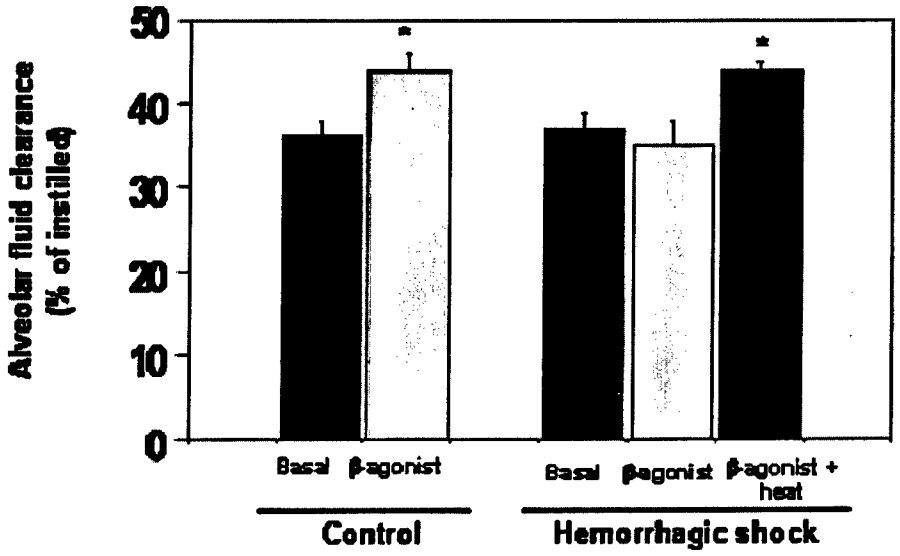

Figure 5. Effects of heat preconditioning on basal and ß-agonist-stimulated alveolar fluid clearance in rate after haemorrhagic shock Hemorrhagic shock abolishes the beta-agonist-mediated stimulation of alveolar fluid clearance in rats. Heat preconditioning reestablishes the normal ability of beta-agonists to stimulate transepithelial sodium and water transport (adapted from 14).

CLINICAL STUDIES

In contrast to cardiology (4, 24), clinical studies examining the role of heat shock proteins and/or chemical chaperones in the patho-physiology of pulmonary diseases are very sparse. In one study, alveolar macrophages from patients with ARDS spontaneously expressed large amounts of HSP70, suggesting a link between stress proteins and lung inflammation in humans (10).

More recently, 4 - phenylbutyric acid (PBA, a low-molecular weight fatty acid) has been shown to have chaperone-like activities, and when administered in patients with cystic fibrosis, PBA improved the apical surface CFTR function, as evidenced by a small but significant increase in nasal potential difference (20, 41). No data exist so far, concerning the possible role of PBA in the treatment of pulmonary diseases associated with impaired alveolar fluid clearance.

HSPs IN PATIENTS SUFFERING FROM SEVERE TRAUMA

Although typically regarded as intracellular proteins, it has recently been reported that heat shock proteins are released from cultured cells. For example, HSP60 and HSP72 have been detected in the plasma of healthy human subjects. In addition to being involved in the modulation of the immune system or serve as antigen carriers for antigen presenting cells, circulating HSPs could also represent a marker of the degree of stress experienced by the organism. Alternatively, circulating HSPs may indicate the ability of the stressed organism to conveniently respond to such a stress.

Consistent with the latter hypothesis, Hsp 72 can be detected in the serum of severely traumatized patients within 30 minutes after injury, and high initial serum levels of Hsp 72 (> 15 ng/mL) were associated with improved survival (13). This could suggest that either trauma survivors have an increased ability to respond to stress, and/or that increased HSP expression may confer protection against severe trauma and its complications.

SUMMARY AND FUTURE DIRECTIONS

Forty years after Ritossa's observation in the Drosophila fly that cells respond to stress by increasing the expression of genes coding for a certain class of cytoprotective proteins, it is know well established that this stress response plays an important role in cardiovascular diseases in humans. Recent data suggest that HSPs may also exert protective effects in the lung.

Preliminary data suggest that chemical chaperones may be useful for the treatment of cystic fibrosis. More importantly, the stress response markedly decreases mortality rates and attenuates cellular insults in several models of acute lung injury and sepsis, suggesting that in the near future induction of the stress response may represent a novel therapeutic tool also for the prevention and/or treatment of pulmonary edema associated with ARDS or heart failure.

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Chapter 20

PROTEINS INVOLVED IN SALVAGE OF THE MYOCARDIUM

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Abstract: In the Western world, cardiac ischemic disease is still the most common cause of death despite significant improvements of therapeutic drugs and interventions. The fact that the heart possesses an intrinsic protection mechanism has been systematically overlooked before the 1980s. It has been clearly shown that the activation of this mechanism can reduce the infarct size after an ischemic insult. Prerequisite is the induction of the synthesis of such cardio-protective proteins as heat shock proteins (HSPs) and anti-oxidative enzymes. HSPs are involved in the maintenance of cell homeostasis by guiding the synthesis, folding and degradation of proteins. Besides, the various family members cover a broad spectrum of anti-oxidative, anti-apoptotic and anti-inflammatory activities. Although the major inducible HSP72 has received most attention, other HSPs are able to confer cardioprotection as well. In addition, it seems that there is a concerted action between the various cardio-protective proteins. One drawback is that the beneficial effects of HSPs seem to be less effective in the compromised than in the normal heart. Although clinical studies have shown that there is a therapeutic potential for HSPs in the compromised heart, major efforts are needed to fully understand the role of HSPs in these hearts and to find a safe and convenient way to activate these protective proteins.

Key Words: heat shock proteins, anti-oxidative proteins, preconditioning, maintained cardio-protection.

INTRODUCTION

Extensive research has been undertaken to delay the onset and reduce the extent of myocardial cell damage during and after an ischemic insult or other stressful cardiac event. Although good results have been obtained using exogenous pharmacological measures such as vasodilators and calcium-antagonists, less attention has been paid to the fact that

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the heart itself possesses one of the most powerful measures of protection.

Already in 1986, it was shown that brief periods of reversible ischemia limited the infarct size caused by a subsequent prolonged period of ischemia (47). Although originally observed in dogs, this phenomenon, called ischemic preconditioning, was rapidly shown to exist in other animals such as the swine (58), rabbit (5), rat (37) and mouse (44). A major disadvantage is that this "early" protection has a relatively short duration, i.e. usually less then 120 min after the trigger coronary occlusion (54) (see Figure 1).

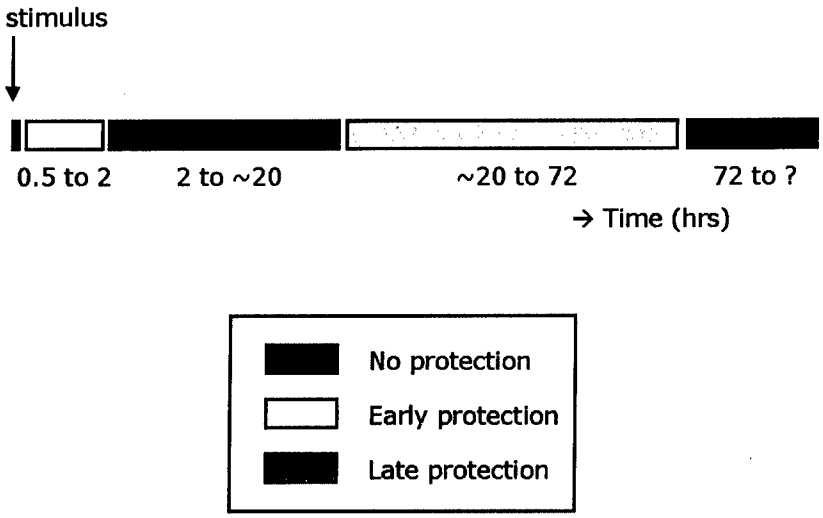

Figure 1. Scheme depicting the two temporal phases of cardioprotection observed after an ischemic stimulus. Cardioprotection is usually observed as a decreased infarct size after a prolonged ischemic episode. The early protection is more potent than the late protection. The second phase of protection can also be induced by numerous other stimuli such as heat and heavy exercise (see text).

Intriguingly, there is also a "second" window of protection afforded by the same initial ischemic stimulus. This delayed or "late" myocardial protection becomes apparent about 24 hours later (32, 40) and lasts for about 2 to 3 days (5). In this case the second window of myocardial protection is induced by ischemia (i.e. delayed phase of ischemic preconditioning). However it can also be elicited by other stresses like heat-shock or endotoxin exposure (59). It has been shown that the second window of protection coincides with the expression of the so-called stress proteins or heat shock proteins (HSPs) and anti-oxidative proteins and compounds like nitric oxide (NO).

This review will focus on the delayed cardioprotective phase and will discuss the possible mechanisms responsible for this phenomenon. Furthermore, it will explain possible routes to this protection and will point out the implications for the (pathological) human situation. Finally, considerations for future research are presented.

INITIATORS OF THE (HEAT) STRESS RESPONSE

Myocardial protection, attributed to enhanced levels of cardioprotective proteins, can be induced by numerous general stressors, such as ischemia/reperfusion, heat shock, endotoxins, rapid cardiac pacing, exercise and many pharmacological treatments (for reviews see (9, 59)). Without doubt a very complicated cascade of intracellular messengers is activated, the search on which is very active. To pinpoint the exact initiator leading to the whole gush of intra- and extra-cellular changes seems, however, a difficult task (see for overview Figure 2).

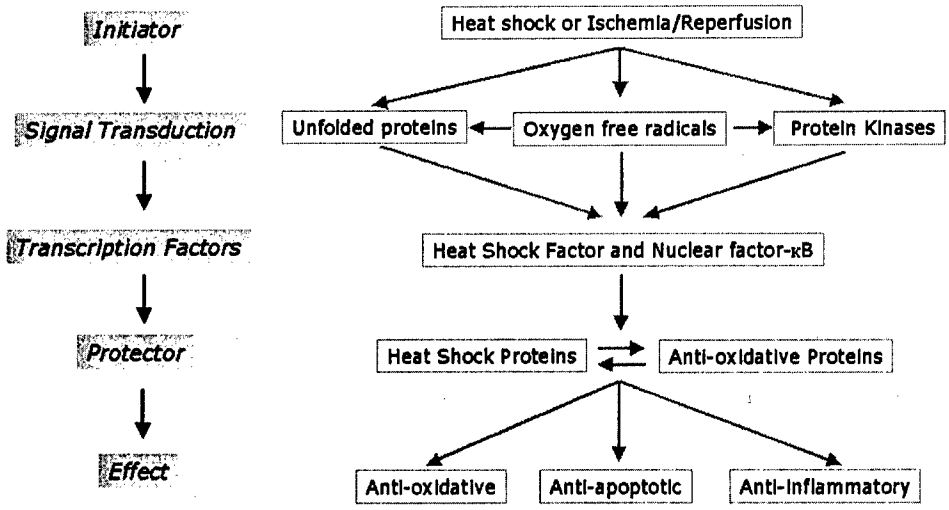

Figure 2. The delayed phase of protection is attributed to the novo synthesis of cardioprotective proteins. Most stresses increase the number of (partially) denatured proteins, generate oxygen free radicals and activate protein kinases (such as protein kinase C). These intermediates act together and activate different transcription factors such as the heat shock transcription factor and nuclear factor kappa B. Binding of these transcription factors lead to transcription and subsequent translation of such cardio-protective proteins as heat shock proteins or anti-oxidative proteins. These sentinels influence each other and thereby cover a broad spectrum of anti-oxidative, anti-apoptotic and anti-inflammatory activities.

Protein Denaturation

In a general way all the above-mentioned types of stress have the same central effect in living organisms: protein denaturation inside the cell. It has been shown that the destabilization of proteins can serve as triggers for hsp-gene activation (4). Support for this hypothesis comes from the observation that chemical stabilization of proteins by glycerol or D_2O before application to stress blunts the hsp-gene expression (23). Through the loss of their normal conformation, proteins expose their hydrophobic regions, and allow specific HSPs (e.g. HSP72 see below) to bind. The binding of HSP72 to partially unfolded proteins leads

to the release of the heat shock transcription factor (HSF), which is normally bound to HSP72. Thereafter, HSF can form homo-trimers and translocate to the nucleus where they bind to the heat shock responsive elements (HSE) that are present in the promoter region of most HSP-genes and initiate their transcription (46). Upon stress recovery, HSP72 hydrolyzes ATP and undergoes conformational changes to correctly refold the affected proteins.

Mechanical Deformation

In several studies it has been demonstrated that increased preload (stretch) also induces HSF activation (48) and subsequent hsp72 mRNA synthesis (19, 30). As such, it can be appreciated that myocardial ischemia dilates or stretches the left ventricle (28). Whether this is also true for other inducers of the heat shock response remains unclear. It might be possible that other factors like increased contractility and/or release of hormones may influence the activation of HSFs. It is also known that the HSF-DNA binding and subsequent transcription and protein translation of HSPs can be enhanced by a combination of two stressors (25). At present it is unknown whether the mechanical stimulus applied is associated with protein unfolding.

Other Mediators

Mitochondrial dysfunction is often associated with such stresses as sepsis and ischemia. For example, ATP-production is decreased and reactive oxygen species are increased by ischemia (65,77). Adenosine is a breakdown product of ATP and may be released during stress. Its involvement in the protective mechanism has been implicated using adenosine receptor agonists and antagonists (7). Another candidate is nitric oxide (NO) (11). All the above-mentioned candidates probably have an intricate relationship with each other, as exemplified by studies that link the activation of the adenosine receptor to the nitric oxide synthase (NOS) pathway in delayed cardioprotection after ischemic preconditioning (63,76).

SIGNALING PATHWAYS

With regard to the signal transduction pathways, it seems that delayed stress preconditioning is associated with the activation of protein kinase C (6, 69), mitogen-activated protein kinases (75) and the tyrosine kinases (18). It should be mentioned that the involvement of one kinase system does not exclude other kinase systems since close interactions exist between the different kinase cascades (52). Downstream these kinases may activate transcription factors, such as HSFs or nuclear factor kappa B (68) that are implicated in the delayed cardioprotection (Figure 2). Moreover, there is reason to believe that these different transcription factors co-operate to correctly express the proper cardioprotective proteins (57).

CARDIOPROTECTIVE PROTEINS

Heat Shock Proteins

The HSPs comprise a group of highly conserved proteins. Some of these are abundantly expressed and have diverse functions, including the assembly and sequestering of multi-protein complexes, transportation of nascent polypeptide chains across cellular membranes and regulation of protein folding (24). Also called molecular chaperones, HSPs fall into five general families, based on their molecular weight: HSP110, HSP90, HSP70, HSP60, and the small molecular weight HSP families. Although these families comprise several members, only the typical representatives of each family will be briefly discussed.

HSP110: In the heart the members of this family have not yet been well investigated. Their synthesis is increased upon stress and these proteins are involved in proteolytic pathways.

HSP90: HSP90 forms a complex with the steroid hormone receptors rendering the non-ligand bound receptor transcriptionally inactive. Its localization can either be cytosolic or nuclear. HSP90 also guides proteins to the cellular membrane.

HSP60: HSP60 can be localized in the mitochondrion and cytosol. Its function, which is stimulated by HSP10, is to guide mitochondrial import of proteins. HSP60 is also involved in cardiac apoptosis.

Small HSPs: This group contains several important members, i.e. HSP32 and HSP27. HSP27 is active in assisting the assembly of macroglobular protein complexes, such as that of F-actin. Furthermore, it protects microfilaments from disruption and aggregation. HSP32 is the rate-limiting enzyme in the degradation of heme to biliverdin, molecular iron, and carbon monoxide. HSP32 has broad protective effects such as in wound healing and in oxidative stress.

HSP70: With regard to myocardial protection, members of the HSP70 family (especially HSP72) are by far the most investigated HSPs. Evidence is at hand that the major inducible HSP upon stress, i.e. HSP72 plays a central role in cellular protection. Hyperthermic treatment, leading to HSP72 overexpression has been shown to reduce infarct size (15, 22). In addition, a correlation was observed between the amount of HSP72 induced by heat pretreatment and the degree of myocardial protection (27). In transgenic mice constitutively expressing the human or rat HSP72 protein, overabundance of the protein was associated with protection of myocardium from ischemia and reperfusion injury without apparent negative side effects (41, 53).

In the last 5 years more attention has been paid to other HSPs. Experiments were mostly performed on isolated myocardial cells but they show that HSP27, HSP60 alone or in combination with HSP10 (36) or HSP90 (14) could confer protection against (simulated) ischemia. The strong protective role of HSP32 or heme-oxygenase against oxidative stress is emerging more and more (43, 72, 73). As indicated above, heme-oxygenase is categorized into the family of HSPs since an HSF-binding site was discovered in its promoter region. Upon enhanced expression, heme-oxygenase exerts a cardioprotective effect via its powerful ant-oxidative activity. As such it can be linked to another class of endogenous protective proteins, i.e. the anti-oxidative proteins (Figure 2).

Anti-Oxidative Proteins

Various forms of stress also generate harmful reactive oxygen species. The heart responds to these free radicals by enhancing the activity of anti-oxidative enzymes. Among others, it has been shown that both hyperthermia and ischemia increase the activity of manganese superoxide dismutase (Mn-SOD; primarily present in the mitochondria) and catalase (70, 71).

Is seems that there is interplay between the different groups of cardioprotective proteins. In a recent paper by Suzuki and coworkers, it was postulated that the enhanced Mn-SOD activity during ischemia-reperfusion injury is a possible mechanism of HSP72-induced cardioprotection (61).

As mentioned earlier severe exercise can also enhance the expression level of HSPs (56). This property has been linked to the increased myocardial tolerance to ischemia and reperfusion damage (56). But, short-term exercise in a cold environment still improves post-ischemic myocardial function without the induction of HSP72 (67) or other HSPs (26). The exercise-induced cardioprotection however was always associated with an increase in myocardial antioxidant defenses (Mn-SOD). These findings might implicate that the exercise-induced overexpression of HSPs is rather a stress indicator than an intrinsic protective mechanism.

A simplified scheme, which addresses the different steps involved in the upregulation of the cardioprotective proteins, is presented in Figure 2.

Nitric Oxide Synthase

NOS has been demonstrated to be a protective protein, through the vaso-active activity of its products (10). The heart is equipped with three different isoforms of NOS. The inducible NOS, iNOS is found in most cardiovascular cells including the cardiomyocyte and is highly inducible upon stress, while the endothelial cells contain the constitutive eNOS. Finally, n(euronal)NOS is found in neurons in the heart. NOS generates nitric oxide (NO), which is responsible for maintaining vasodilatation of the coronary vessels and numerous other effects. The eNOS isoform is regarded as one of the triggers for the delayed window of protection after an ischemic stimulus whereas the iNOS isoform is responsible for early cardioprotection. The latter was demonstrated using a pharmacological or genetic approach (10). But it has to be mentioned that excessive amounts of NO have detrimental effects on both short and long term survival and myocardial function after ischemia and reperfusion (especially when large amounts of reactive oxygen species are present).

(Heat) stress leads to increased NO-levels, which precedes the HSP72 induction (39). The release of trigger NO by eNOS is regulated, among others, by HSP90, which further implies an intricate relationship between the various protective proteins.

HSPS IN THE DELAYED PHASE OF ISCHEMIC PRECONDITIONING

The involvement of HSPs in the delayed phase of preconditioning is still unclear. It is well known that brief periods of ischemia/reperfusion induce HSP-overexpression (29). Therefore, the second window of protection observed after ischemic preconditioning has initially been attributed to the elevated levels of HSP72 and/or HSP60 (40) (32). However, other studies using a less severe ischemic preconditioning protocol, did not confirm the association of presence of protection and enhanced synthesis of HSP72. Therefore, it could be that HSP72 –when present- is merely a marker of ischemic stress. Up to now, the precise role of HSP72 and the other HSPs in the second window of protection is not yet fully elucidated.

CELL-TYPES INVOLVED IN DELAYED CARDIOPROTECTION

It has now been well established that enhanced levels of HSPs reduce infarct size after ischemia and reperfusion and thereby significantly improve cardiac function. But do we know which cell types are the central targets for this protection? Volume-wise, cardiomyocytes occupy the vast majority of the cardiac mass. However, they are by far outnumbered by non-muscle cells like fibroblasts, endothelial cells and smooth muscle cells. Below we summarize the findings on the various HSP functions in the separate cardiac cell-types.

Cardiomyocytes

Numerous studies have shown that isolated (adult or neonatal) cardiomyocytes have the property to enhance the level of HSPs upon heat or ischemic stress, and that this overexpression confers protection (for reviews see (9, 59)). In addition, immunohistochemical evaluations in intact hearts have shown that HSP72 is expressed in cardiomyocytes after ischemia and reperfusion (3, 74).

Endothelial Cells

Several authors have suggested that endothelial cells might be crucial for the HSP-mediated cardioprotection (51). Regarding the stress response, it was shown that after an *in vivo* stress protocol, endothelial cells expressed much higher levels of HSP72 than did cardiomyocytes (35). Furthermore, when normal endothelial function was impaired, myocardial protection against ischemia and reperfusion was totally lost (3). Although eNOS seems not to be implicated in the delayed phase of protection after an ischemic episode, adenoviral gene transfer of eNOS was shown to reduce the extent of *in vivo* ischemia-reperfusion injury in the rat heart (2).

Smooth Muscle Cells

Vascular smooth muscle cells form the contractile portion of the walls of arteries and are highly influenced by paracrine mediators of other cell-types. For example, the role of NO in mediating smooth muscle relaxation is well established. It is postulated that HSPs (especially the small HSPs) mediate vasorelaxation by directly modulating cytoskeletal or contractile elements in muscle cells. In addition, overexpression of SOD in coronary vascular cells (endothelium and smooth muscle cells) of transgenic mice renders them more resistant to ischemia and reperfusion damage (12).

Fibroblasts

Cardiac fibroblasts are crucial in the maintenance of myocardial and vascular structural integrity and play a pivotal role in remodeling in diseased hearts. The fibrillar collagen network mainly establishes myocardial integrity. HSP47 is considered to be a collagen-specific molecular chaperon expressed by fibroblasts. Its expression is closely related to that of collagen, as was observed during the remodeling process after cardiac infarction (64). The formation of collagenous fibrous tissue is a vital part of the process of wound healing. Recently it was shown that also HSP32 has an important role in this process. The different aspects of the wound healing process after myocardial infarction correlated well with the content of HSP32 (33).

In summary, it seems that every cardiac cell type has a specific pattern of HSP expression (type and level) after stress. Besides, each of these HSPs and other cardioprotective proteins can be associated with a protective action in the various cell types. Although several studies show that overexpression of HSPs in specific cell types is protective *in vivo*, it is anticipated that optimal defense against stress is a balance between these cell types.

PROTECTION IN THE COMPROMISED HEART

In the healthy aged heart the stress response is less powerful than in the healthy adult heart. This is illustrated by the lower HSP72 levels after heat stress (13) or ischemia (49). In addition, the ischemia tolerance is diminished in the aged heart. As such, some controversy exists on the protective effects of HSPs in the aged heart. Locke and coworkers showed that heat stress pretreatment induced the upregulation of HSPs in 22 months old rats, although this could not be associated with protection (38). In contrast, however, our own group has shown that the aged heart (18 months) can be preconditioned by heat stress against ischemic damage (13). Differences in the animal strain, ischemic protocol and experimental circumstances (42) might play a role in this controversy. The healthy adult but hypertrophied heart is as capable as the normal heart to express enhanced levels of HSP72 after stress (13). In contrast the senescent hypertrophied but still compensated heart has lost, to a large extent, its ability to express increased HSP72 content after *in vivo* heat shock. However, despite this impairment the senescent hypertrophied heart can still be protected against the deleterious effects of ischemia and subsequent reperfusion (13).

With regard to the chronically failing heart as a consequence of infarction, it has been shown that hyperthermia-induced upregulation of HSP72/73 was partly blunted. This was associated with depressed function during hyperthermia (66). These results suggest that functional deterioration of the failing heart upon stress can possibly be attributed to a reduction in the production of myocardial HSP72.

With respect to the other protective proteins, cardiac hypertrophy is associated with an increase in antioxidant capacity. However, in the failing heart the anti-oxidative enzyme (i.e. SOD) activities were lower (21). As such the combination of a deteriorating anti-oxidative defense system and a reduced stress response in the failing heart coincides with the decreased tolerance to ischemic damage.

The role of NO in cardioprotection in the compromised heart is controversial. The increased activity of myocardial iNOS plays a negative role in the development of postischemic cardiac dysfunction and injury in the (hypertensive) hypertrophic heart. The hypertrophied heart has lower myocardial sensitivity to NO and a lower bioactivity of NO (51).

MAINTAINED CARDIOPROTECTION

It has been shown that the second window of protection has a limited duration and only lasts 2 to 3 days (5). For optimal effectiveness of this mechanism, hearts with high risks for infarction should be put into a permanent preconditioned state. The ultimate goal is to put hearts with high risks of coronary occlusion in a permanent preconditioned state. In one study, a non-toxic preparation from gram-positive bacteria has been shown to induce long-term cardioprotection (50). Protection against ischemia/reperfusion damage remained present up to 21 days after the initial injection and was associated with increased activity of catalase and higher expression of HSP72. Attempts to induce long-term protection against stunning consisted of repeating the preconditioning stimulus (60). It was found that the positive anti-stunning effects could be associated with enhanced HSP72 levels. Modest but regular alcohol consumption as well can induce a maintained protection against ischemia/reperfusion injury, probably through activation of adenosine A_1 receptors (45). Therefore, pharmacological approaches were also investigated. Dana and co-workers showed that the rabbit heart could be put in a long-term protected state against myocardial infarction by repeated (every 48 hours for 5 days) activation of adenosine A1 receptors (16). This is most likely to be associated with mitochondrial Mn-SOD activation (17). Long-term endurance training in animals was also found to be associated with cardioprotection, which remained present up to 5 days after finishing the training program. (for review see (56)). Again a reduction was found in myocardial oxidative injury after *in vivo* ischemia/reperfusion, which was probably related to an increased activity of Mn-SOD (20, 55). Finally, gene transfer leading to a long-term enhanced expression of human HSP32, a known anti-oxidant, produces a long-term (8 weeks) myocardial protection, as shown by a dramatic reduction in infarct size after myocardial ischemia (43). As these findings are still limited in number, further research is needed in order to complete the image of whether these approaches are clinically relevant.

CLINICAL APPLICABILITY OF PRECONDITIONING

Most of the animal studies on protective proteins and preconditioning have been performed in healthy hearts. The methods described in these experiments proved to be very potent in rendering the heart less susceptible to the effects of ischemia. However, most of them (transient ischemia, heat shock or endotoxin exposure) are not readily applicable in the clinical setting. Moreover, the induced protective effect is transient. This currently limits the clinical application of preconditioning to anticipated and well-controlled situations in which myocardial ischemia or hypoperfusion might occur (e.g. revascularisation procedures, cardio-thoracic surgery, transplantation). Despite these limitations, the evidence of the possibility to put the human heart in this protected state is increasing. In accordance with the observations from animal experiments, different methods to induce cardioprotection in the human heart have been described. Induction of the acute phase of cardioprotection has been achieved by temporary aortic cross-clamping (62) and administration of isoflurane (8) prior to cardiac surgery.

The "second window of protection" has been demonstrated to be inducible in patients experiencing preinfarct angina. This process has been shown to be much less effective in the senescent heart, although a high level of physical activity is associated with preservation of the cardioprotective effect of preinfarction angina in elderly patients (1).

To the best of our knowledge, there are no studies demonstrating pharmacological or interventional induction of the second window of cardioprotection in humans.

Because of the positive effects observed in laboratory animals and patients in both the first as well as in the second "windows of protection", further studies with the goal to induce protection in a safe and practically applicable way are indicated.

In the future, the increasing possibilities of genetic manipulation might be of additional value. Hypothetically, lowering the threshold needed for the induction of cardioprotection or increasing the expression of protective proteins by genetic manipulation could be another way to decrease the detrimental effects of ischemia and reperfusion.

CONCLUSIONS

The normal heart is equipped with a number of protective proteins that can be called upon in times of need. The most important proteins are the heat shock proteins and enzymes involved in the detoxification of oxygen free radicals. The expression pattern (and functionality) of these cardioprotective proteins is divergent in the compromised heart (31, 34). This indicates that more efforts should be undertaken to characterize and to unravel the mechanisms of these endogenous measures in the compromised heart. Only then, we can specifically attack the complex nature of myocardial injury after ischemia and reperfusion or other stresses in this type of heart. Thereafter, gene therapy can become clinically relevant. "Pre-event" gene therapy approach is potentially beneficial to patients with chronic coronary artery disease undergoing coronary intervention or cardiac surgery. "Post-event" gene therapy might be beneficial, since HSPs are also involved in repair processes, like wound healing. It is anticipated that these novel molecular biological approaches have great potential even on the long run.

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Chapter 21

THE NO - K^+ CHANNEL AXIS IN PULMONARY ARTERIAL HYPERTENSION

Activation by experimental oral therapies

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Abstract: The prognosis of patients with pulmonary arterial hypertension (PAH) is poor. Available therapies (Ca^{++}-channel blockers, epoprostenol, bosentan) have limited efficacy or are expensive and associated with significant complications. PAH is characterized by vasoconstriction, thrombosis in-situ and vascular remodeling. Endothelial-derived nitric oxide (NO) activity is decreased, promoting vasoconstriction and thrombosis. Voltage-gated K^+ channels (Kv) are downregulated, causing depolarization, Ca^{++}-overload and PA smooth muscle cell (PASMC) contraction and proliferation. Augmenting the NO and Kv pathways should cause pulmonary vasodilatation and regression of PA remodeling. Several inexpensive oral treatments may be able to enhance the NO axis and/or K^+ channel expression/function and selectively decrease pulmonary vascular resistance (PVR). Oral L-Arginine, NOS' substrate, improves NO synthesis and functional capacity in humans with PAH. Most of NO's effects are mediated by cyclic guanosine-monophosphate (c-GMP). cGMP causes vasodilatation by activating K^+ channels and lowering cytosolic Ca^{++}. Sildenafil elevates c-GMP levels by inhibiting type-5 phosphodiesterase, thereby opening BK_{Ca} channels and relaxing PAs. In PAH, sildenafil (50mg-po) is as effective and selective a pulmonary vasodilator as inhaled NO. These benefits persist after months of therapy leading to improved functional capacity. 3) Oral Dichloroacetate (DCA), a metabolic modulator, increases expression/function of Kv2.1 channels and decreases remodeling and PVR in rats with chronic-hypoxic pulmonary hypertension, partially via a tyrosine-kinase-dependent mechanism. These drugs appear safe in humans and may be useful PAH therapies, alone or in combination,

Key Words: pulmonary hypertension, potassium channels, redox, sildenafil, gene therapy

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INTRODUCTION

PAH - A Model Vascular Disease

Pulmonary Arterial Hypertension (PAH) is a disease of the pulmonary vasculature, defined by an elevated pulmonary vascular resistance (PVR), which eventually leads to heart failure and premature death. Fifty years after its original antemortem description (27), although there is still no cure for PAH, much has been learned recently about its etiology. In PAH, the pulmonary arteries (PA) are affected, in varying degrees, by excessive vasoconstriction, vascular remodeling (including distal extension of PA muscularization, cellular proliferation in both the intima and the media, plexiform lesions) and thrombosis in situ. All of these changes lead to narrowing or obliteration of the PA lumen, increase in the right ventricular afterload and failure of the afterload intolerant right heart.

A "multiple hit" hypothesis has been proposed for the pathogenesis of PAH, similar to that proposed for the pathogenesis of neoplasia, in which exogenous stimuli (e.g. exposure to the anorectic drugs or HIV infection) lead to the development of PAH in genetically predisposed individuals (2). Genetic abnormalities have now been described in patients with familial PAH (10-20% of all PAH cases), as well as in those with sporadic PAH (126). Several loss-of-function mutations have been described in receptors of the transforming growth factor-beta (TGF-β) superfamily, such as bone morphogenetic protein receptor II (BMPR2) (26, 54, 127) or activin receptor-like-kinase 1 (ALK1) (128). These receptors are linked to the SMAD second messenger system, an important regulator of gene transcription involved in cell proliferation and apoptosis (125). Activation of the TGF-β BMPR2 axis leads to suppression of proliferation and activation of apoptosis; conversely, these loss-of-function mutations exaggerate the susceptibility of vascular cells to proliferate. Indeed, proliferating endothelial cells which form the plexogenic lesions (25) in PAH have been shown to be monoclonal (58). Even in the absence of germ line mutations, endothelial cells microdissected from plexogenic lesions display microsatellite instability (acquired mutations) within the human MutS Homolog 2 gene that lead to reduced protein expression of TGF-β and thus suppression of apoptosis and monoclonal growth of "neoplastic" endothelial cells (146). Furthermore, PA smooth muscle cells (PASMC) from BMPR-knockout mice have abnormally enhanced proliferation rates in response to growth factors in vitro (82). Similar abnormalities on the TGF-BMP pathways have been described in tumors, like the juvenile colonic polyposis (44), or vascular lesions such as the coronary restenosis lesions post angioplasty (63). The extensive diversity and tissue specificity of the SMAD system and the heteromultimer formation of different TGF/BNP receptor subtypes (9), may explain the restriction of the vascular disease to the pulmonary circulation in patients with PAH and BMPR2 mutations.

DNA microarray studies have shown downregulation of several genes associated with apoptosis and upregulation of genes associated with cell proliferation in PAH lungs, as would be expected from the inhibition of the BMP pathway (35). The same studies showed that genes for the voltage-gated potassium channels (Kv) are downregulated in PAH, in contrast with the genes for inward rectifier potassium channels (Kir) (35). This is in agreement with earlier reports that showed that specific Kv channels, like Kv1.5 or Kv2.1, are downregulated in the PASMC in humans with PAH (148) as well as in rats with chronic hypoxia-induced pulmonary hypertension (CH-PHT) (73). Kv channels like Kv1.5

and Kv2.1 control PASMC membrane potential (7) and therefore the activity of the L-type voltage-gated Ca^{++} channels. The selective loss of these Kv channels leads to PASMC depolarization, opening of the L-type Ca^{++} channel, influx of Ca^{++}, increase in the $Ca^{++}i$ and vasoconstriction (75). Whether these Kv channel abnormalities are genetically determined or acquired (for example, anorexigens, such as dexfenfluramine, that caused epidemics of PAH are Kv channel inhibitors (74)) is unknown. It is also unknown whether these PASMC Kv channel abnormalities are related to the BMPR2 abnormalities that have been described mostly in PA endothelial cells (146).

A fascinating mechanism for a potential "cross-talk" between the PASMC and endothelial cells has recently been described in PAs from patients with both familial and sporadic PAH. The expression of angiopoietin-1, a protein involved in the recruitment of SMC around blood vessels, is significantly enhanced in PASMC from patients with PAH compared to controls (28). Interestingly, angiopoietin-1 shuts off the expression of BMPR1A, a transmembrane protein required for BMPR2 signaling, in pulmonary arteriolar endothelial cells in vitro (28).

Several other abnormalities have been described in PAH. There is a constrictor/dilator imbalance in the endothelium (decreased NO and prostacyclin and increased endothelin-1 and thromboxane). In addition, platelets have abnormal serotonin handling and plasma serotonin levels are increased (for review see (2))

In summary, the vasoconstriction of the PAs in PAH can be explained by the loss/inhibition of PASMC Kv channels and the decreased endothelial-derived vasodilatation. The vascular remodeling appears to be due, in part, to dysfunction of BMP-driven apoptosis. Until recently, the treatment of PAH has focused on drugs that target vasoconstriction pathways. For example, the L-type Ca^{++} channel blockers, effective in the ~20% of patients, (103) target the PASMC Ca^{++} channels that are activated because of the depolarization caused by the downregulation of Kv channels. Epoprostenol (Flolan™) (13) replaces depressed levels of endogenous prostacyclin. The recently introduced nonspecific endothelin receptor antagonist, bosentan, targets the upregulated production of endothelin-1 by PAH endothelial cells. The recently described abnormalities in cell proliferation pathways will undoubtedly lead to the development of therapies that specifically target vascular remodeling. However, it is likely that some of the "vasodilator" strategies are in fact beneficial because chronically they also promote regression of vascular disease.

The Profile of the Ideal Candidate Treatment for PAH

An ideal candidate therapy or cocktail of therapies for PAH should lead to both vasodilatation and regression of vascular remodeling. However, most of the disordered pathways in PAH are important in regulating systemic vascular tone. Several treatments of PAH are often limited by their lack of selectivity, for example enhancement of the NO axis or inhibition of the Ca^{++} channels is often limited by systemic hypotension. Of the ~20% of all PAH patients that show a favorable acute response to vasodilators, only a small fraction can be effectively treated with large required doses of dihydropyridines, because of systemic side effects. To overcome this challenge, differences between the pulmonary and systemic circulation need to be considered in the design of therapeutic approaches in order to achieve selectivity for the pulmonary circulation.

The quality of life in patients with PAH is compromised. They usually are diagnosed

long after the onset of symptoms, often when they are functionally class III-IV. The only therapy that has been shown to prolong life in these patients, is continuous epoprostenol infusion, which is therefore considered the gold standard (13). Because of its extremely short half life, continuous administration via portable intravenous pumps is required, further compromising the quality of life and adding the risk of infection to the already long list of significant side effects of this drug. Moreover, epoprostenol costs ~ CAN $100,000/ year. Bosentan is administered orally, but its effectiveness is limited (patients on bosentan walk only 43 meters longer in a 6 minute walk compared to patients on placebo), and it not infrequently causes a dose-dependent liver toxicity (107). Bosentan costs ~ CAN$55,000/ year.

The ideal drug for PAH should be oral, safe, well tolerated and affordable. This review will discuss 3 candidate treatments that have the potential to meet several of these standards. They target the NO (L-Arginine, sildenafil) and the K^+ channel axis (dichloroacetate) in the pulmonary circulation. We will first discuss several differences between the pulmonary and systemic circulation that might explain the selectivity of these treatments. The NO axis and the role of K^+ channels will be discussed then, followed by a discussion of the effects of these drugs in both animals and humans with pulmonary hypertension.

THE PULMONARY VERSUS THE SYSTEMIC CIRCULATION

The normoxic pulmonary circulation is low pressure and constricts to hypoxia (Hypoxic Pulmonary Vasoconstriction, HPV) while the systemic circulation is higher pressure and dilates to hypoxia. HPV is an evolutionary conserved mechanism for optimizing the matching of ventilation and perfusion. HPV is mediated, in part, by a redox mechanism (71, 137). Recently significant redox differences have been described between the pulmonary and systemic vascular beds (like the renal circulation) (72). The pulmonary circulation is in a more oxidized redox state compared to the renal circulation as reflected by the higher levels of activated oxygen species (AOS, superoxide and hydrogen peroxide) and compensatory increase in glutathione (GSH) (72). These differences might be in part due to the fact that the PA smooth muscle cells (SMC) have functionally different mitochondria compared to renal artery SMC. The PASMC mitochondria are more depolarized, and have higher levels of mitochondria manganese superoxide dismutase, presumably to compensate for their greater production of AOS. These differences may reflect the different ambient PO_2 to which the vascular beds, specifically the resistance arteries, are exposed (140mmHg for PAs versus <80mmHg for the renal circulation). Such differences in the redox O2 sensor may explain in part the opposing effects of hypoxia on the pulmonary and renal circulations (72).

These redox differences might also be important in the interpretation of the role of several redox-sensitive second messengers and pathways in the vascular biology of the two circulations (for review see (141)). One very important redox-sensitive pathway is the NO axis. NO is itself a radical (141). There are significant differences in the NO axis between the pulmonary and systemic circulation, and between the healthy and diseased pulmonary circulation, as discussed subsequently. These differences need to be taken into account when considering the effects of possible treatments for PAH that enhance the endogenous NO axis, like sildenafil and L-Arginine.

Furthermore, a reflection of the mitochondrial diversity between the pulmonary and systemic circulation might also be the fact that the mitochondrial enzyme pyruvate dehydrogenase kinase (PDK) type 2 is expressed at higher levels in the lungs versus the heart and peripheral muscles (20). This might explain in part why dichloroacetate, a PDK inhibitor, which enhances the function and expression of K^+ channels and reverses pulmonary hypertension in rats (106), has beneficial pulmonary but not systemic hemodynamic effects in pulmonary hypertension as will be discussed below (73).

THE NO AXIS IN THE VASCULATURE

NO is formed by oxidation of a terminal guanidino nitrogen of L-Arginine in the presence of a heme-containing enzyme, NOS (79). The process is oxygen-dependent and important cofactors include reduced nicotinamide adenine nucleotide phosphate, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin (79). There are 3 known NOS isozymes: Isozyme I is mostly expressed in neurons (neuronal NOS, nNOS) but also in epithelial and vascular cells including PASMC (114). Isozyme II is induced (inducible NOS, iNOS) by several mediators of inflammation and is regulated at the level of gene expression. Once expressed, iNOS produces NO at very high rates. In contrast to isozymes I and III, the activity of iNOS is independent of the levels of intracellular Ca^{++}. Isozyme III is constitutively expressed mostly in, but not exclusively, in endothelial cells (endothelial NOS, eNOS). Although eNOS is the main isozyme involved in the regulation of vascular tone, both nNOS and iNOS have been reported to be involved in the production of pulmonary vascular NO, both in disease states and during normal development. (19; 95)

Whereas at very high levels NO reacts with superoxide, giving rise to potentially toxic substances like peroxynitrite, at lower levels, as it occurs within the normal vasculature, NO activates soluble guanylate cyclase, resulting in increased levels of cyclic guanosine monophosphate (cGMP) within the target cells (79). cGMP activates a cGMP-dependent protein kinase (PKG), which is responsible for most of the vasodilatory effects of NO (79). A major pathway by which NO relaxes PAs is via cGMP-kinase-dependent phosphorylation of PASMC sarcolemmal potassium (K^+) channels (Figure 1) (5; 105).

K^+ CHANNELS IN THE PULMONARY CIRCULATION

K^+ channels are transmembrane proteins with a pore-forming unit that allows the selective efflux of K^+ ions from the cytoplasm (6). Based on electrophysiologic, pharmacologic and molecular criteria, K^+ channels in the vasculature are separated into 3 families: voltage-gated, Kv, Ca^{++}-activated (KCa) and inward rectifier (Kir) (8). When K^+ channels open there is an efflux of K^+ ions from the cells down a concentration gradient (intracellular / extracellular K^+ concentration = 140 / 5 mEq) and the interior of the cell becomes more negatively charged (hyperpolarization). In contrast, when K^+ channels close, the PASMC depolarizes. Depolarization beyond a threshold (~-40mV) increases the opening of the voltage-gated, L-type Ca^{++} channels, leading to influx of Ca^{++}, activation of the actin-myosin complex and contraction (8). K^+ channels in the endothelium may also be important in

regulating the activity of NOS and participating in the endothelial derived hyperpolarizing factor pathway. Thus, in blood vessels, SMC K^+ channel openers are vasodilators and SMC K^+ channel inhibitors are vasoconstrictors. Of course, not all channels that are present are open at resting membrane potential and inhibiting a class of channels only alters tone if the channel was previously open. Conversely, K^+ channel openers cause relaxation even if the targeted channel was not previously a participant in setting resting membrane potential. For example, NO causes vasodilatation in part by opening the large conductance KCa channels (BK_{Ca}), which are normally quiescent in a healthy PASMC. This leads to PASMC hyperpolarization, inhibition of the voltage-gated L-type Ca^{++} channels, a decrease in the intracellular Ca^{++} concentration $[Ca^{++}]_i$; and vasorelaxation (5).

In addition to the effects on K^+ channels, cGMP-kinase causes a decrease in $[Ca^{++}]_i$ via effects on several types of Ca^{++} channels and transporters and the sarcoplasmic reticulum (83). Furthermore, cGMP-kinase causes SMC relaxation by direct effects on the actin-myosin apparatus (143).

Kv channels, like Kv1.5 and Kv2.1, control PASMC membrane potential and their inhibition by hypoxia is important to the initiation of HPV (8; 136). Direct inhibition of these two channels by anorectic agents like dexfenfluramine occurs and may participate in the pathogenesis of anorectic-induced pulmonary hypertension, which has occurred in several outbreaks in recent years (74). Furthermore, a selective downregulation of Kv channels has been implicated in the pathogenesis of chronic hypoxic pulmonary hypertension (73; 149) and in primary pulmonary hypertension in humans (150).

The pathway leading to BKCa channel activation in the pulmonary circulation is shown in Figure 1 (5). cGMP levels are regulated by the balance between production (by soluble and particulate guanylate cyclase) and degradation (by type 5 phosphodiesterase). The activity of the BK_{Ca} channels in the PASMC is additionally regulated by the balance between phosphorylation by the cGMP-kinase (which promotes activation) and the de-phosphorylation by phosphatases (which promote inhibition) (5). This pathway has provided therapeutic targets for the treatment of pulmonary hypertension (Figure 1).

NO AND THE NORMAL PULMONARY CIRCULATION

NO plays a role in regulating tone in the normal pulmonary circulation, but much less that it does in the systemic circulation. In the pulmonary circulation NO is primarily a compensatory vasodilator that increase in the constricted or hypertensive pulmonary circulation to lower pulmonary vascular resistance (PVR). This conclusion is mostly based on experiments using NOS inhibitors, showing that these drugs do not alter tone in a variety of models, including isolated PAs, perfused lungs and intact animals, nicely summarized and reviewed recently by Hampl and Herget (40). This is in contrast to the systemic circulation, where NOS inhibitors routinely increase tone (40) (Figure 2).

These data suggest that under normal conditions, a basal tonic release of NO from the endothelium regulates vascular tone in the systemic but not the pulmonary circulation. There are several possibilities that could explain this difference. First, expression of eNOS is low in normal resistance PAs, the vessels that essentially control PVR, and most of the eNOS is seen in the endothelium of the large-conduit PAs in both animals (48; 57; 130; 145) and humans (51). This is in contrast to pulmonary hypertension, where strong

expression of eNOS is seen in the resistance and neomuscularized PAs (Figure 3) (57; 130; 145), as discussed later. Second, the biological effects of NO are modulated by the redox environment of the target cell, as NO itself is a radical which is rapidly oxidized in the presence of O_2 (3). In addition to differences in NO production, the significant differences in the redox environments might further differentially regulate NO levels in pulmonary vs. systemic circulations.

The fact that eNOS knockout mice develop mild pulmonary hypertension, compared to wild mice, at first might suggest that NO plays a role in controlling baseline pulmonary vascular tone in this model (122). However, if the eNOS function is inhibited by NOS inhibitors in the wild control mice, they do not develop significant pulmonary hypertension. It has been postulated that the fact that the eNOS -/- mice have mild pulmonary hypertension reflects an abnormal transition from the fetal to the neonatal pulmonary circulation (40). In contrast to the low-pressure adult pulmonary circulation, NO appears to be important in the control of tone in the high-pressure fetal pulmonary circulation (118; 119). In other words, NO contributes to the normal transition from the fetal to the low-pressure neonatal pulmonary circulation and the lack of eNOS at this critical transition, results in persistence of the fetal remodeling and increased resistance in the pulmonary circulation. Therefore, the pulmonary hemodynamics in adult eNOS-/- mice do not reflect lack of NO in the pulmonary circulation. This is further supported by the fact that they do not decrease their PVR in response to exogenous NO (40). Alternatively, there may be altered expression of other important enzymes, including other NOS isoforms, in these mice.

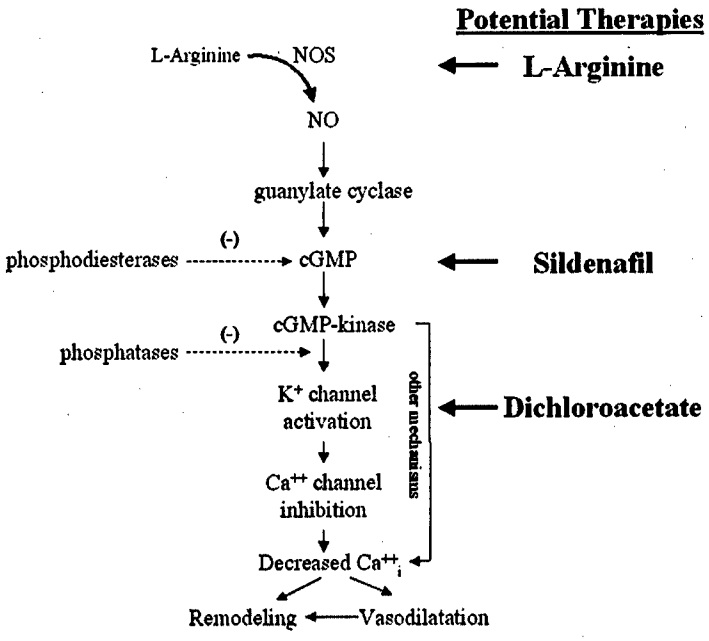

Figure 1. The NO axis and potential therapeutic targets in the treatment of pulmonary hypertension.

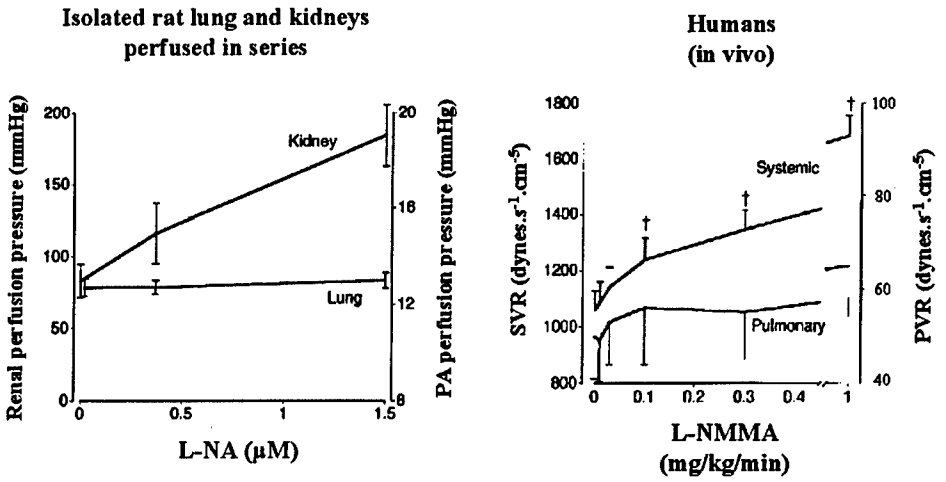

Figure 2. NOS inhibitors increase systemic more than pulmonary vascular resistance in isolated rat organs (left) and in humans (right).Obtained with permission (40).

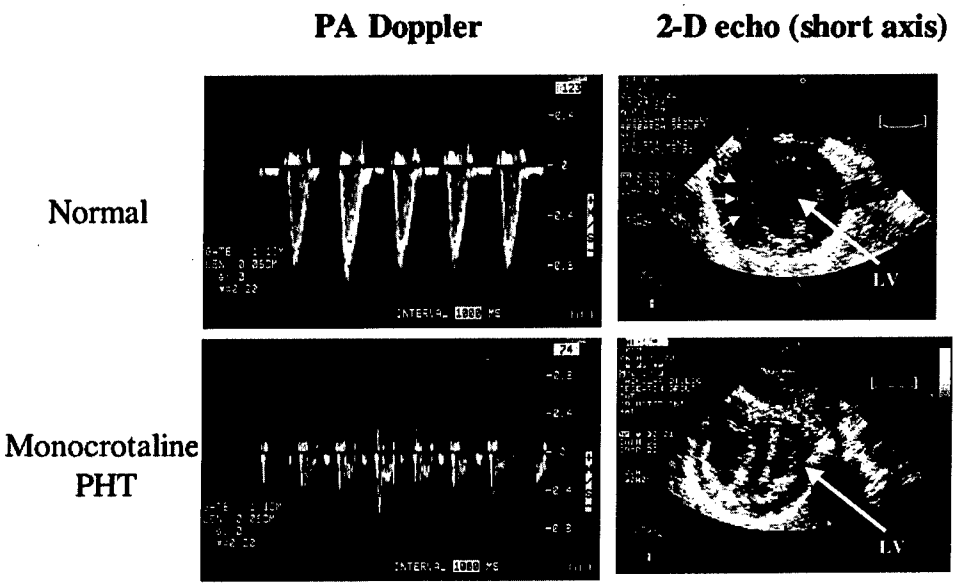

Figure 3. The development of pulmonary arterial hypertension in the rat monocrotaline model as seen by echocardiography. Note that with monocrotaline pulmonary hypertension there is flattening of the interventricular septum (arrows), compressing the left ventricle into a "D" shape. In addition, the pulmonary artery Doppler signal shows a shortened PA acceleration time and systolic notching. These changes are identical to those seen in human PAH.

THE NO AXIS IN PULMONARY HYPERTENSION

The importance of NO in the pathogenesis or maintenance of PHT varies between species, different models of PHT and different stages of the disease. The NO axis is rarely assessed comprehensively within a single study (i.e. NOS mRNA, NOS protein expression, NOS activity, NO and NOx levels) and this further complicates the assessment of the role of NO on this disease.

Pulmonary Hypertension: Animal Models and Human Disease

There are 3 commonly used animal models of pulmonary hypertension.
1) Chronic hypoxia-induced pulmonary hypertension (CH-PHT). This model is relevant to the PHT in humans with chronic obstructive pulmonary disease (41). Rats develop pulmonary hypertension in 1-3 weeks of placement in a hypoxic chamber (10% O2).
2) Monocrotaline-induced pulmonary hypertension (MC-PHT). In this model rats develop severe pulmonary hypertension after a single intraperitoneal dose of monocrotaline, an alkaloid found in the weed crotalaria spectabilis. Monocrotaline is thought to initiate pulmonary hypertension via its toxic effects on the endothelium, but the mechanism remains unknown (78; 109; 131). The increase in right ventricular afterload leads to RVH, with echocardiographic features similar to those found in patients with PAH (Figure 4).

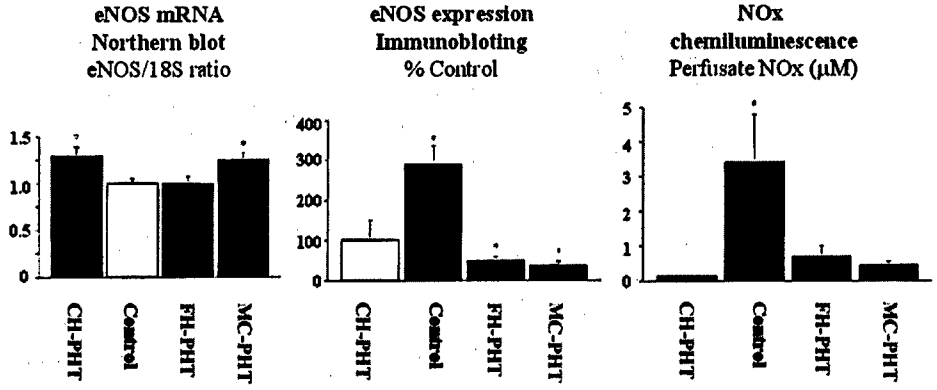

Figure 4. Comparison of eNOS mRNA levels, protein expression and activity in lungs from rats from 3 different pulmonary hypertension models. The findings are discussed in the text and show the importance of using multiple complementary techniques in the study of the NO axis in pulmonary hypertension. Obtained with permission from (130).

3) The Fawn-hooded rat. These rats develop spontaneous pulmonary hypertension and although they have a platelet disorder and abnormal serotoninergic metabolism, the

pathogenesis of pulmonary hypertension in this model also remains unknown (55; 56; 120).

Human PAH includes all forms of pulmonary hypertension, except those that are secondary to thromboembolic pulmonary vascular disease, parenchymal lung disease and secondary to abnormalities of the pulmonary veins, mitral valve or left ventricle. The term PAH encompasses primary pulmonary hypertension (PPH), in which there is no association of the elevated PVR with an identifiable cause, as well as pulmonary hypertension associated with the use of anorectic agents, rheumatologic diseases (like scleroderma or lupus erythematosus), congenital heart disease, HIV infection and cirrhosis (portopulmonary hypertension) (2).

While not significantly expressed in the normal resistance PAs, eNOS expression is increased in the endothelium of the resistance PAs in CH-PHT (57; 100; 130; 145), MC-PHT (100; 130) and the FH rats (130). How eNOS expression is altered in human PAH remains unclear. Gaid and Saleh reported decreased eNOS expression (37), whereas Xue and Johns (144) reported increased and Tuder et al. (129) noted unaltered eNOS immunostaining. The discrepancies amongst the human studies and between the human versus animal data are likely due to methodological differences as well as differences in the stages and severity of the disease in the models studied. For example, animals tend to be studied early in the development of the disease, whereas human lungs tend to be studied at the end stages of the disease; biopsies are now rarely performed in the workup of pulmonary hypertension and most of the tissue is obtained during transplant surgery or postmortem. Antigen retrieval can be problematic in pathology specimens obtained at autopsy, resulting in false-negative immunohistochemistry.

Increases in the expression of NOS do not necessarily imply increase in the NO production, since the increased protein might have decreased enzyme activity. For example, Rengasamy et al. (98; 99) using the citrulline assay, showed that the activity of eNOS is decreased under hypoxic conditions, whereas as discussed above, the protein expression of this enzyme is often increased in chronic hypoxia. These authors suggest that O2 substrate limitation might regulate NOS activity under hypoxic conditions (98; 99). NOx accumulation in the perfusate is significantly elevated in the lungs isolated from rats with CH-PHT (46) (130). In the later study, the investigators compared eNOS mRNA, protein expression and activity (NO, NOx levels) in all 3 models of rat pulmonary hypertension (Figure 3) (130). They showed that while mRNA for eNOS was increased in both the CH-PHT and MC-PHT and was unaltered in the FH rats, protein expression was increased in the CH-PHT rats but was decreased in FH and MC-PHT rats (130). Lung perfusate NOx increased in the CH-PHT rats but was unchanged in the FH and MC-PHT rats, although there was a trend towards an increase in NOx in the MC-PHT rats (130) (Figure 3). We have found that NO counteracts effects of anorexigens (Figure 5). And exhaled NO is increased in some but not all forms of PAH (Figure 5).

The mechanism for the eNOS upregulation in experimental remains unclear. At least for CH-PHT it is possible that Hypoxia Inducible Factor (HIF) induces NOS, since HIF-1 expression is increased by hypoxia in PASMC and endothelial cells (147). However, there is yet no evidence for a HIF-1 binding site in the human eNOS promoter, in contrast to iNOS (69) and nNOS (31).

There is now evidence for crosstalk between NO and ET-1 through an autocrine feedback loop (52). For example, in endothelial cells ETB receptor activation stimulates

eNOS activity (42) (142). Therefore, it is possible that ET-1, which is known to be elevated in several models of pulmonary hypertension and in humans (32; 38) (123) is in part responsible for the eNOS upregulation. On the other hand, NO-cGMP inhibits ET-1 secretion and gene expression (52). NO donors, such as molsidomine have been shown to inhibit the formation of ET-1 in the pulmonary circulation of rats with CH-PHT (16).

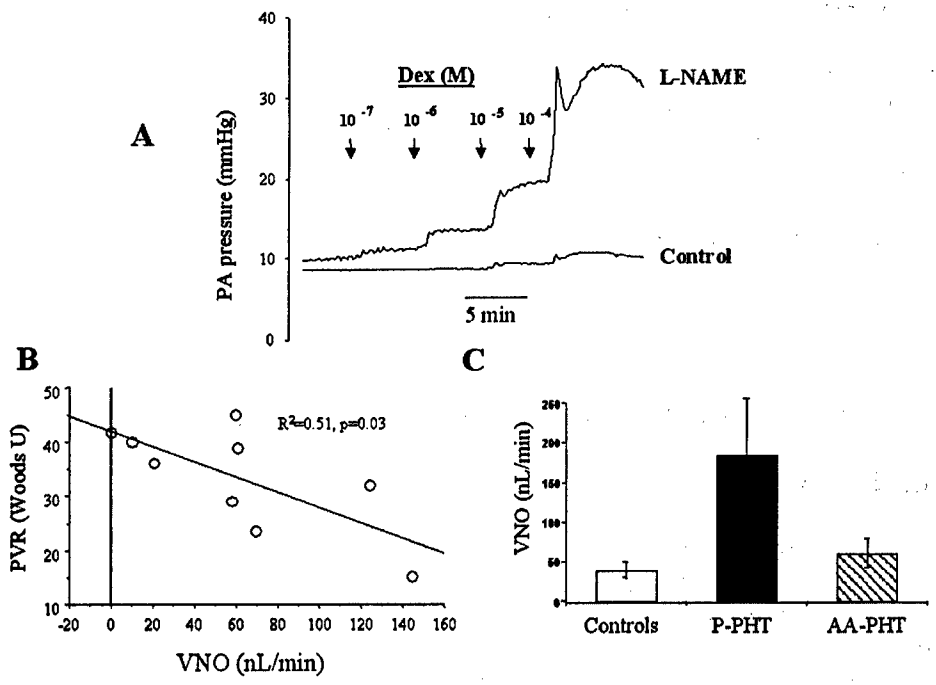

Figure 5. A. Dexfenfluramine (Dex) significantly increases PVR in an isolated, perfused rat lung, but only after the lung is pretreated with a NOS inhibitor. Note that NOS inhibition minimally increases the baseline tone in this lung from a healthy rat. Obtained with permission (138). B and C. NO production VNO (controlled for minute ventilation) correlates inversely with the PVR in patients with PAH associated with prior anorexigen exposure. Patients with anorectic-associated pulmonary hypertension (AA-PHT) have lower VNO levels compared to the elevated levels seen in patients with primary pulmonary hypertension (P-PHT). This suggests that perhaps low NO levels in the pulmonary circulation of AA-PHT (due to endothelial dysfunction) predisposed them to the development of PHT after the ingestion of anorectic agents.Obtained with permission from (4).

The NO axis might also be important in the remodeling of the PAs in pulmonary hypertension since NO inhibits SMC proliferation (34; 112) and induces apoptosis in vascular SMCs (33; 89). The role of apoptosis in the development of the PA remodeling in pulmonary hypertension is not clear. Does remodeling require increased apoptosis or does apoptosis promote regression from remodeling and medial hypertrophy? Also, it is possible that apoptosis in the endothelial cells is different than the apoptosis in the smooth muscle cells in the PA media. Nevertheless, Yuan et al. recently showed that NO might induce PASMC apoptosis by activating Kv and KCa channels as well as depolarizing

mitochondrial membrane potential ($\Delta\Psi m$) (149). The opening of sarcolemmal K^+ channels would cause efflux of K^+ and therefore osmotic cell shrinkage, which is known to initiate apoptosis; $\Delta\Psi m$ depolarization has also been implicated in the initiation of apoptosis (29). This elegant study raises the intriguing possibility that the loss of Kv channels, associated with the development of pulmonary hypertension in animals (73; 75) and humans (150), might contribute to the development of PA remodeling by suppressing a physiological level of PASMC apoptosis, thus permitting PASMC proliferation, resulting in medial hypertrophy and neomuscularization.

The role of the NO axis in the development and maintenance of pulmonary hypertension remains unclear and eNOS knockout models have not offered a definitive answer. In contrast to Steudel et al. (122) and Fagan et al. (30), Quinlan et al. (94) found that eNOS deficient mice show decreased muscularization and media thickness in resistance PAs in response to chronic hypoxia, compared to the control mice. They speculated that differences in the genetic background of the eNOS deficient and control mice might have accounted for the opposing results between their study and those from the other groups.

EXOGENOUS ENHANCEMENT OF THE NO AXIS

Inhaled NO (iNO)

Exogenous iNO can reach the PASMC of resistance PAs via diffusion through the alveoli (62; 121). After further diffusion into the lumen, NO reacts with hemoglobin and is inactivated, avoiding any systemic effects. Furthermore, iNO will only be delivered in ventilated lobes and thus dilate only the vascular beds in well ventilated areas. The lack of vasodilatation in nonventilated areas will prevent the intrapulmonary shunting seen with the systemically administered pulmonary vasodilators and preserve V/Q matching.

Initiation of chronic therapy for PAH usually follows an acute hemodynamic trial to determine prognosis, assess safety of a proposed treatment and guide future medical therapy (101-103; 115). The acute hemodynamic study employs a pulmonary vasodilator, usually either a short-lived substance which is nonselective (adenosine or prostacyclin) or a selective pulmonary vasodilator (iNO), to evaluate the responsiveness of the pulmonary vasculature while minimizing systemic hypotension (87; 101; 102; 115). iNO is currently considered the gold standard for the evaluation of patients with PAH (87; 101; 102; 115). A positive response to iNO (> 20% decrease in pulmonary artery pressure or pulmonary vascular resistance) predicts a positive response to conventional vasodilators, such as calcium channel blockers (101; 115) and identifies patients with a better long-term prognosis than the non-responders (103). iNO is also extensively used in the short-term treatment of neonatal pulmonary hypertension (1).

The chronic use of inhaled NO is limited by its short half-life and, more recently, significant increases in the price of this gas. Even its use as an acute vasodilator is cumbersome, requiring an expensive medical form of NO gas, a complicated delivery system and monitoring equipment. Nevertheless there is some preliminary evidence that chronic outpatient therapy is possible. In an uncontrolled pilot study of chronic iNO in 5 PAH patients, using nasal cannulae and a gas pulsing device, improvement in PA

pressure or cardiac output was shown after 12 weeks of treatment in 3 out of the 5 patients (23). Chronic continuous exposure to iNO significantly prevents monocrotaline-induced remodeling in the pulmonary circulation in rats (104).

There are 2 important potential complications of even the short-term use of iNO, pulmonary edema and rebound pulmonary hypertension upon discontinuation of iNO. First, iNO causes increase in the pulmonary artery wedge pressure, especially in patients with left ventricular dysfunction (60; 110), perhaps explaining occasional cases of pulmonary edema with this therapy (17). It has been suggested that this is a result of the increased return of blood from the lungs to a noncompliant left ventricle. Recently, however pulmonary edema was reported in patients with PAH due to the CREST syndrome with normal left ventricular function (93). Pulmonary artery wedge pressure was increased by iNO in a cohort of 11 patients with PAH and 2 patients with left ventricular dysfunction (70). In this study, wedge pressure was not increased in response to sildenafil, a phosphodiesterase inhibitor despite a similar decrease in PVR and a greater increase in cardiac index compared to iNO (70). Both iNO and sildenafil (Viagra) caused similar increases in the cGMP in the pulmonary circulation (70).

Sudden termination of iNO occasionally causes a potentially life threatening hypertensive rebound. This can occur after even a few hours treatment and has bee reported in patients that showed no initial vasodilator response (76). This may be explained by the fact that exogenous NO can decrease in eNOS activity (15; 113) and increase endothelin levels (66). In addition to its potent vasoconstrictor effect, endothelin induces superoxide production, which in the presence of NO causes the formation of peroxynitrite (135). This suggests that endothelin receptor blockers might be beneficial in the management iNO-rebound effect (135).

Another way of delivering NO selectively in the pulmonary circulation is using inhalation of aerosolized adenoviruses carrying the genes for eNOS or iNOS (21; 47) or using cell-based gene transfer of eNOS (22). These approaches are very promising forms of gene therapy but several challenges need to be overcome before their human application, such as the immune reactions against the adenovirus and the transient nature of the expression of the transferred gene (< 1 month for adenovirus).

ENHANCEMENT OF THE ENDOGENOUS NO AXIS

L-Arginine

It has been suggested that the production of NO can be limited by insufficient supply of NOS substrate, i.e. L-Arginine. Therefore L-Arginine has been given in a variety of cardiovascular diseases, in an attempt to optimize NO production. For example, oral L-Arginine improves endothelial function and exercise capacity in patients with congestive heart failure (96). Intraperitoneal injections of L-Arginine have also been shown to reduce mean PA pressure, PA remodeling (% muscularization) and right ventricular hypertrophy in both rats with CH-PHT and MC-PHT (77) (Figure 6A). Mehta et al. showed that in humans with both primary and secondary pulmonary hypertension, intravenous administration of L-Arginine acutely decreases PVR (67). Although systemic vascular resistance was slightly decreased in both the patients and healthy controls, PVR was not decreased in

those patients with congestive heart failure but no pulmonary hypertension, suggesting a relatively selective effect of L-Arginine in the hypertensive pulmonary vasculature. The same group later showed that systemic intravenous L-Arginine increases exhaled NO (68). Oral administration of L-Arginine (0.5g capsule/10Kg body weight) in 12 patients with PAH and 7 with chronic thromboembolic disease acutely decreased PVR by 16% and, after 1 week or treatment, slightly improved exercise capacity compared to placebo (85) (Figure 6B). A small decrease in the systemic arterial pressure was once again noted (from 92(4 to 87(3 mmHg, $p<0.05$). Several small studies were stopped because L-Arginine was reported to cause large decreases in SVR in patients with pulmonary hypertension (Table 1). More studies are needed to establish the role of this drug in the treatment of patients with pulmonary hypertension, especially in combination with other drugs that enhance the NO axis, like sildenafil. However, based on cost and potential efficacy this strategy merits further exploration in a large multicentre trial.

Table 1. Summary of Acute Human Trials of L-Arginine in Adult Pulmonary Hypertension (PHT)
Abbreviations: SS Systemic Sclerosis, PPH Primary Pulmonary Hypertension, VTE Chronic Venous Thromboembolic Disease, IC Ischemic Cardiomyopathy, ASD Atrial Septal Defect

Reference	L-Arginine Dose (Route)	Patients	Sample Size	ΔPVR with L-Arginine
Baudouin *et al.* 1993 (14)	500mg/kg (IV)	SS	5	No significant change
Surdaki *et al.* 1994 (124)	12.63g (IV)	PPH	4	No significant change (trial stopped early due to large decreases in SVR)
Mehta *et al.* 1995 (67)	500mg/kg (IV)	4 IC, 3 PPH, 2 SS, 1 VTE	10	-27.6±5.8% ($p<0.005$)
Boger *et al.* 1996 (18)	30g (IV)	PPH	5	No significant change
Nagaya *et al.* 2001 (85)	0.5g/10kg (PO)	11 PPH, 7 VTE, 1 ASD	19 (10 randomized to L-Arginine)	-16.2±13.8% ($p<0.05$)

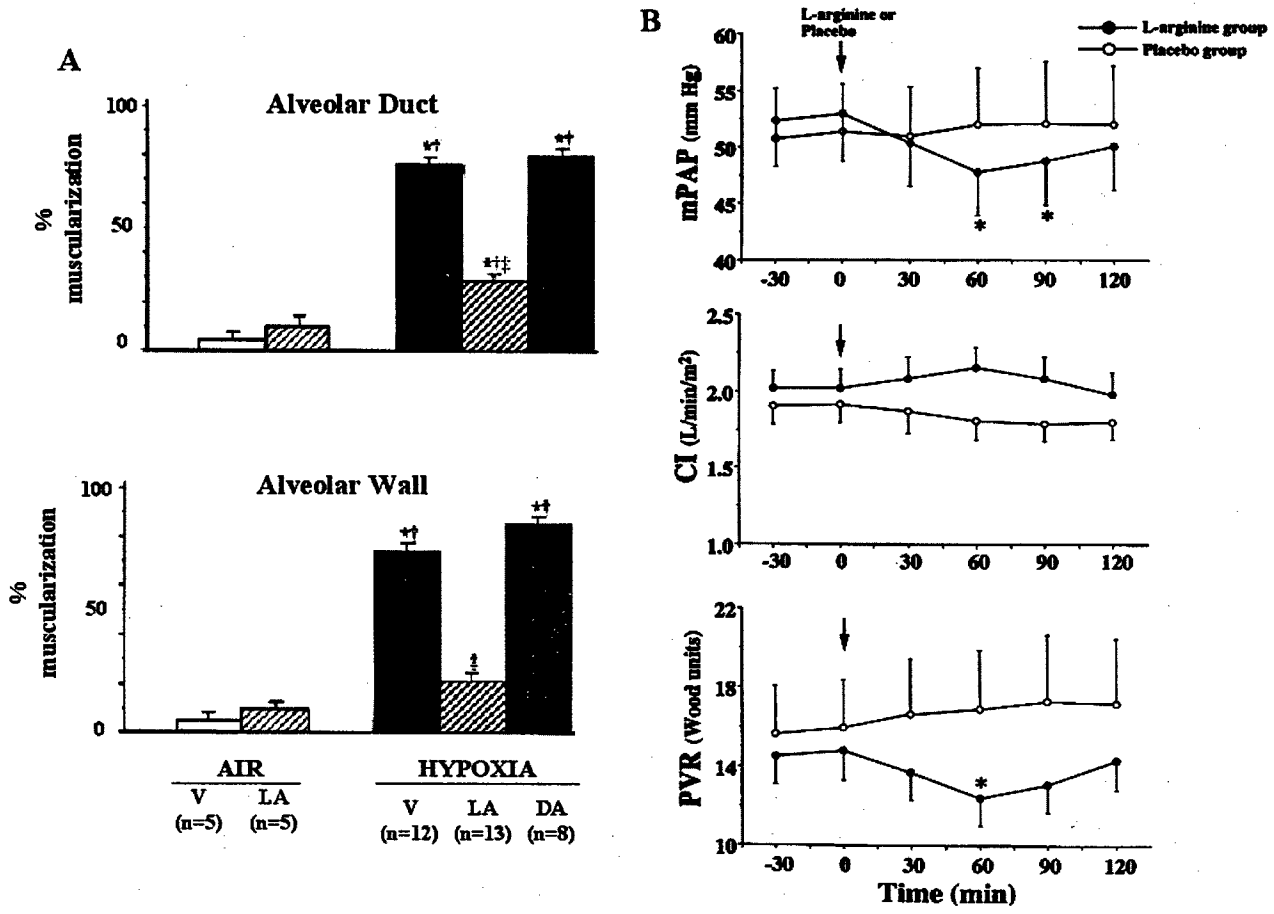

Figure 6. A L-Arginine causes regression of medial hypertrophy in CH-PHT rats. Obtained with permission from (77). B Acute administration of oral L-Arginine (0.5 g/10 kg body weight) or placebo were examined in 19 patients with primary or pre-capillary secondary pulmonary hypertension. Obtained with permission from (85).

Phosphodiesterase Inhibitors

The main effector of NO's vasoactive effects is cGMP, which, like NO, is also short-lived due to the rapid degradation by phosphodiesterases (39) (Figure 1). There are numerous phosphodiesterases but the isoform that is active in degrading cGMP in the lung is phosphodiesterase-5 (108). Phosphodiesterase-5 inhibitors cause pulmonary vasodilatation by promoting an enhanced and sustained level of cGMP, which in turn promotes K^+ channel activation, PASMC hyperpolarization and vasodilatation (Figure 7) (5). There have been recent anecdotal reports and preliminary studies indicating that sildenafil, a specific phosphodiesterase-5 inhibitor widely used in the treatment of erectile dysfunction (24), decreases PVR in humans with primary pulmonary hypertension (PPH) (92; 140), in normal volunteers with hypoxic pulmonary vasoconstriction (151) and in animals with experimental PAH (43; 45). We hypothesized that sildenafil would be as effective in decreasing pulmonary vascular resistance as iNO in the acute assessment of patients with severe pulmonary hypertension. We directly compared the effects of iNO with a single dose of oral sildenafil as well as their combination, on pulmonary and systemic hemodynamics in patients with severe pulmonary hypertension (70).

We studied 13 consecutive patients with a mean (±SEM) age of 44±2 years referred to the University of Alberta Hospital cardiac catheterization laboratory over a period of one year for evaluation of suitability for transplantation or medical therapy. Eleven patients had PAH and two patients had pulmonary hypertension, which although it was associated with left ventricular dysfunction, was disproportionate to their pulmonary wedge pressure. We showed that a single dose of oral sildenafil is a potent and selective pulmonary vasodilator (70). Compared to iNO, sildenafil was superior in decreasing the mean PAP and equally effective and selective in reducing PVR (Figure 8), the primary endpoints of this study (70). In contrast to iNO, sildenafil increased in the cardiac index (Figure 8) and, like iNO, oral sildenafil did not lower the mean systemic arterial pressure (70).

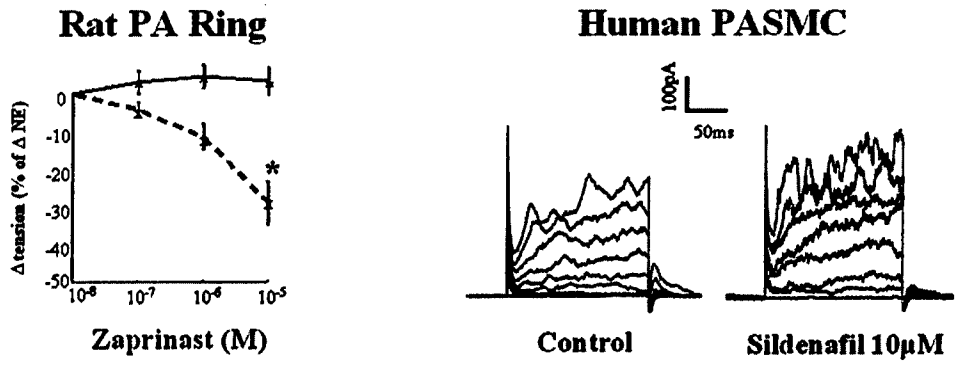

Figure 7. Left: Zaprinast, a preferential phosphodiesterase 5 inhibitor, relaxes the norepinephrine preconstricted rat PA (solid line = vehicle). $P<0.05$ vs. control.Obtained with permission from (5). **Right:** The phosphodiesterase 5 inhibitor sildenafil activates K^+ currents in freshly isolated human PASMC, studied with the whole-cell patch clamping technique. As discussed in the text, this K^+ channel activation explains, at least in part, the pulmonary vasodilatory properties of this drug.

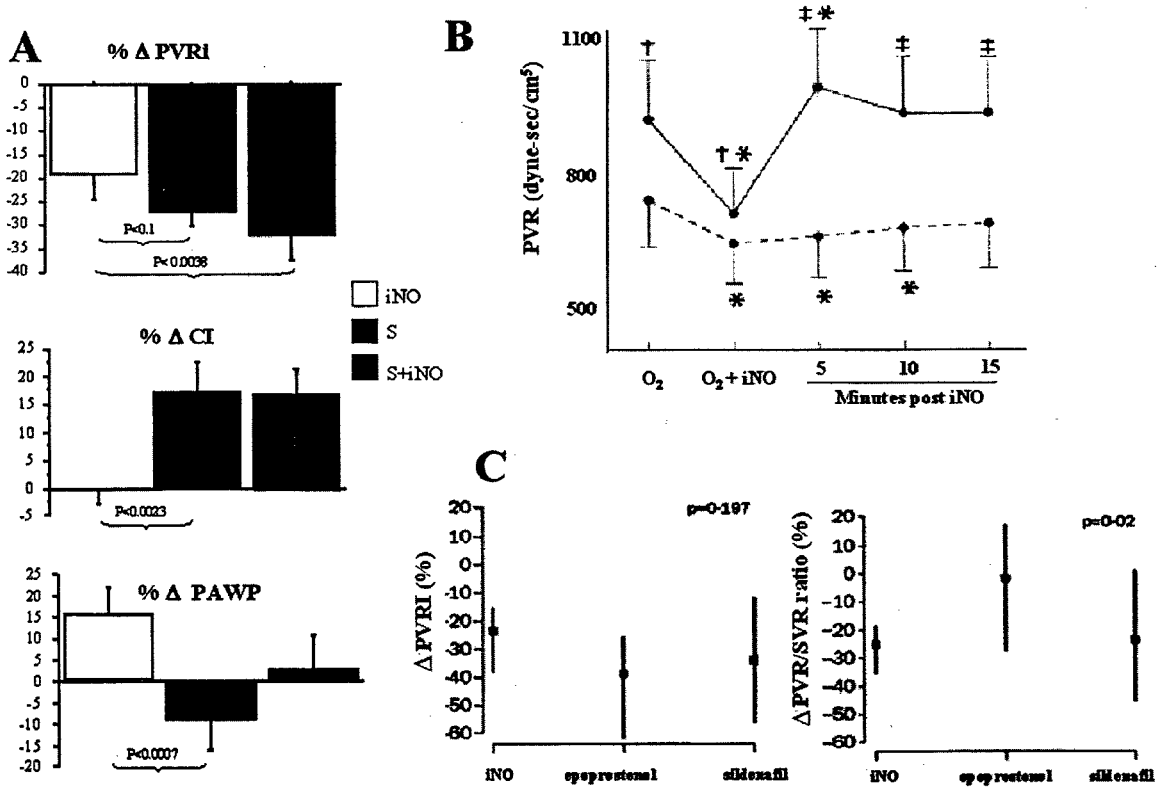

Figure 8. A. Sildenafil and iNO acutely cause similar selective decreases in PVR but only sildenafil increases CI in this cohort of patients with PAH. Obtained with permission from (70). **B.** Effect of sildenafil on the duration of the pulmonary vasodilator response produced by NO inhalation. The PVR (top panel) is shown at baseline (patient breathing O2), during the addition of iNO (O2+NO), and 5, 10, and 15 minutes after cessation of NO inhalation The mean value ± SEM is indicated at each time point during evaluation of the effects of NO inhalation in the absence of sildenafil administration (solid line) or during evaluation of the effects of NO inhalation in combination with oral sildenafil (broken line). Obtained with permission from (59). **C.**. Sildenafil and iNO are more selective pulmonary vasodilators than epoprostenol in patients with pulmonaryhypertension due to pulmonary fibrosis. Obtained with permission from (50).

The finding that sildenafil tends to decrease the wedge pressure (Figure 8) suggests that sildenafil might be superior to iNO in the evaluation of the patients with severe pulmonary hypertension. This might have important safety implications both for the acute study and for eventual long-term use of this drug in patients with left ventricular dysfunction. That oral sildenafil is an effective and selective pulmonary vasodilator in patients with PAH was confirmed by Lepore et al. (59) (Figure 8 B).

The preferential effect of sildenafil on the pulmonary circulation probably reflects the preferential expression of this isoform in the lung. However, phosphodiesterase 5 is also found in the myocardium, where it maybe downregulated in heart failure (111). The finding that sildenafil decreases the wedge pressure and increases the cardiac index suggests that it does not have negative inotropic effects, at least in the patients studied. Phosphodiesterase 5 has been implicated in modulation of sympathetic tone (111) and sildenafil has recently been shown to cause sympathetic nervous system activation in normal volunteers (88). However, the fact that the heart rate did not change after sildenafil in our study suggests that sympathetic activation is not the basis for the observed increase in the cardiac index (70). The data suggest that sildenafil increases cardiac index because of its selective pulmonary vasodilatory effects and the resulting reduction in right ventricular afterload.

The selectivity of sildenafil in the pulmonary circulation was also very recently confirmed by a study showing that sildenafil was very effective in decreasing pulmonary vascular resistance in patients with PHT secondary to lung fibrosis (36). In this study the hemodynamic effects of maximal iNO, epoprostenol and sildenafil (50mg p.o.) were compared. Sildenafil was as effective as iNO and epoprostenol. Moreover, both sildenafil and iNO and were more selective than epoprostenol, as judged by their effects on the PVR/ SVR ratio (36) (Figure 8C).

The simplicity and safety of the acute administration of sildenafil versus iNO and its possible superiority over iNO in terms of its effects on cardiac index and wedge pressure, suggest a role for sildenafil in the evaluation and treatment of patients with pulmonary hypertension and support the need for further studies of its chronic use. Newer phosphodiesterase-5 inhibitors (e.g. tadalafil and vardenafil) that have longer half lives and greater isoform specificity are currently under development (86; 90).

The chronic effects of sildenafil on PAH and specifically the extent of sustained hemodynamic improvement, regression of vascular remodeling as well as improvement in functional capacity, remain to be shown and several studies are currently now under way in both adult and neonatal PAH. A very recent study suggested that indeed sildenafil causes a regression of vascular remodeling in mice with CH-PHT (152). Furthermore this study showed that the regression of remodeling (distal PA muscularization) is in part mediated by Atrial Natriuretic Peptide, since the effects of sildenafil on remodeling were abolished in ANP receptor -/- mice (152) (Figure 9A). Like NO, ANP and Brain Natriuretic Peptide (BNP) raise cGMP (via particulate rather than soluble guanylate cyclase). Interestingly natriuretic peptides have been shown to be related to the severity of PAH (84) and most importantly the decrease in the levels correlate positively with the extent of response to therapy (139) (Figure 9B).

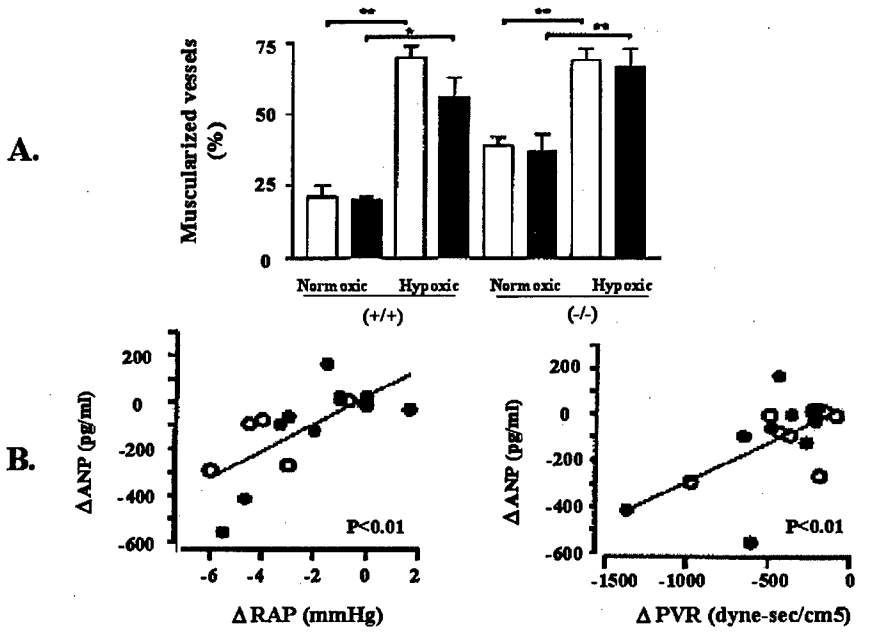

Figure 9. A. The natriuretic peptide pathway influences the response to PDE5 inhibition in hypoxia-induced pulmonary hypertension. Obtained with permission from (152). **B.** The ANP system is highly activated in patients with severe PPH and NPPH. Atrial natriuretic peptide levels are significantly correlated with parameters of RV function and pre- and afterload. Iloprost inhalation causes a rapid decrease in ANP and cGMP in parallel with pulmonary vasodilation and hemodynamic improvement. Obtained with permission from (139).

REVERSAL OF THE DOWNREGULATION OF K^+ CHANNELS IN CH-PHT BY DICHLOROACETATE

DCA inhibits the mitochondrial pyruvate dehydrogenase kinase (PDK) increasing the proportion of the dephosphorylated, active pyruvate dehydrogenase (PDH) (116). The activation of PDH results in an increase in the pyruvate/lactate ratio, promoting an oxidized state (116). In contrast to cardiomyocytes, the energetics of vascular SMC are not well studied. However, it is known that the redox status of vascular SMC (as determined by pyruvate/lactate and NADH/NAD ratios) regulates tone (10; 11) and that DCA increases the pyruvate/lactate ratio in vascular SMC (12).

The ability of DCA to increase the pyruvate/lactate ratio has been used therapeutically in humans. DCA in higher doses has been safely administered orally and intravenously in neonates with lactic acidosis (53; 117) and inborn errors of metabolism (49) and in the treatment of sepsis (132; 133). On the other hand, DCA does not significantly alter the baseline hemodynamics or the exercise performance of patients with stable coronary disease (80; 134). The use of DCA in humans has revealed a good safety profile. There is now great interest in the development of drugs that more potently activate PDH, such as ranolazine (65) or trimetazidine (64). These drugs, known as metabolic modulators, have

been shown to be effective antianginal agents in humans (61).

Of the 4 known PDK isozymes, PDK 2 is the most sensitive to DCA and it is interesting that PDK 2 is preferentially expressed in the lungs, compared to systemic organs, the heart or peripheral muscle (20). Therefore it is possible that potential effects of low dose DCA might be relatively selective for the pulmonary circulation. DCA has been shown to increase the activity of K^+ channels in myocardial cells from infarcted myocardium and these effects were attributed to its metabolic/redox effects since they were mimicked by pyruvate and inhibited by a PDH blocker (106). Thus, we speculated that DCA would reverse the reduced redox state in the PASMC of CH-PHT rats and that this might enhance the activity and expression of Kv channels and reverse CH-PHT, mimicking the benefits of a return to normoxia and without significantly affecting systemic hemodynamics.

Not only are PASMC Kv channels redox sensitive (activated when oxidized and inhibited when reduced (97)) but several transcription regulating genes for specific Kv channels are also redox sensitive (e.g. the Kv repressor element, KRE) (81). This is intriguing since Kv1.5 has been directly involved in the pathogenesis of PAH as discussed above. Kv1.5 has been found to be selectively downregulated in PPH in humans and in animals with PHT, and its replacement using adenoviral mediated gene therapy, causes reversal of PHT in rats (91). This suggests that Kv downregulation is etiologically associated with the pathogenesis of PAH and thus possible reversal of the suppressed Kv activity and expression would reverse CH-PHT.

Indeed, DCA both prevents and reverses established CH-PHT (Figure 10). DCA's beneficial effects are associated with its electrophysiological effects. When given acutely, DCA restores the 4-AP-sensitive component of IK in freshly isolated PASMC from rats with CH-PHT. DCA has no effect on normoxic PASMCs (Figure 10) (73). Very low dose DCA (1μM) activates Kv2.1 expressed in CHO cells by a mechanism involving tyrosine kinase, since it is inhibited by genistein(73), suggesting an additional metabolic-independent mechanism of action or a greater sensitivity of PDK2 to DCA.

When given chronically, DCA reverses the chronic hypoxia-induced downregulation of Kv2.1. These are likely not nonspecific effects since other channels were not affected and indeed there was also a trend for upregulation of the expression of BK_{Ca} channels (Figure 10) (73). Furthermore chronic administration of DCA in the drinking water of rats with CH-PHT reverses and prevents the hemodynamic changes in CH-PHT (73). Direct high fidelity measurement of PA pressure and LVEDP with simultaneous measurement of the CO showed that DCA decreases PA pressure (Figure 10) and PVR, without altering left ventricular end diastolic pressure or systemic arterial pressure. More importantly, DCA decreases the medial hypertrophy of small PAs seen in CH-PHT (Figure 10) (73), despite ongoing hypoxic exposure. Whether this remodeling and regression is a result of DCA's hemodynamic effect or relates to an antiproliferative property of the drug is under investigation.

Our findings that DCA and Kv1.5 gene therapy improves CH-PHT by reversing the changes in both the function and expression of Kv channels, supports a potential causal role for K^+ channel deficiency in the pathogenesis of this form of experimental PHT. We propose that PHT may, in part, be a "K^+ channelopathy". DCA is a very attractive drug to be studied in human PHT, particularly as it has already been used in small, acute human studies without major toxicity.

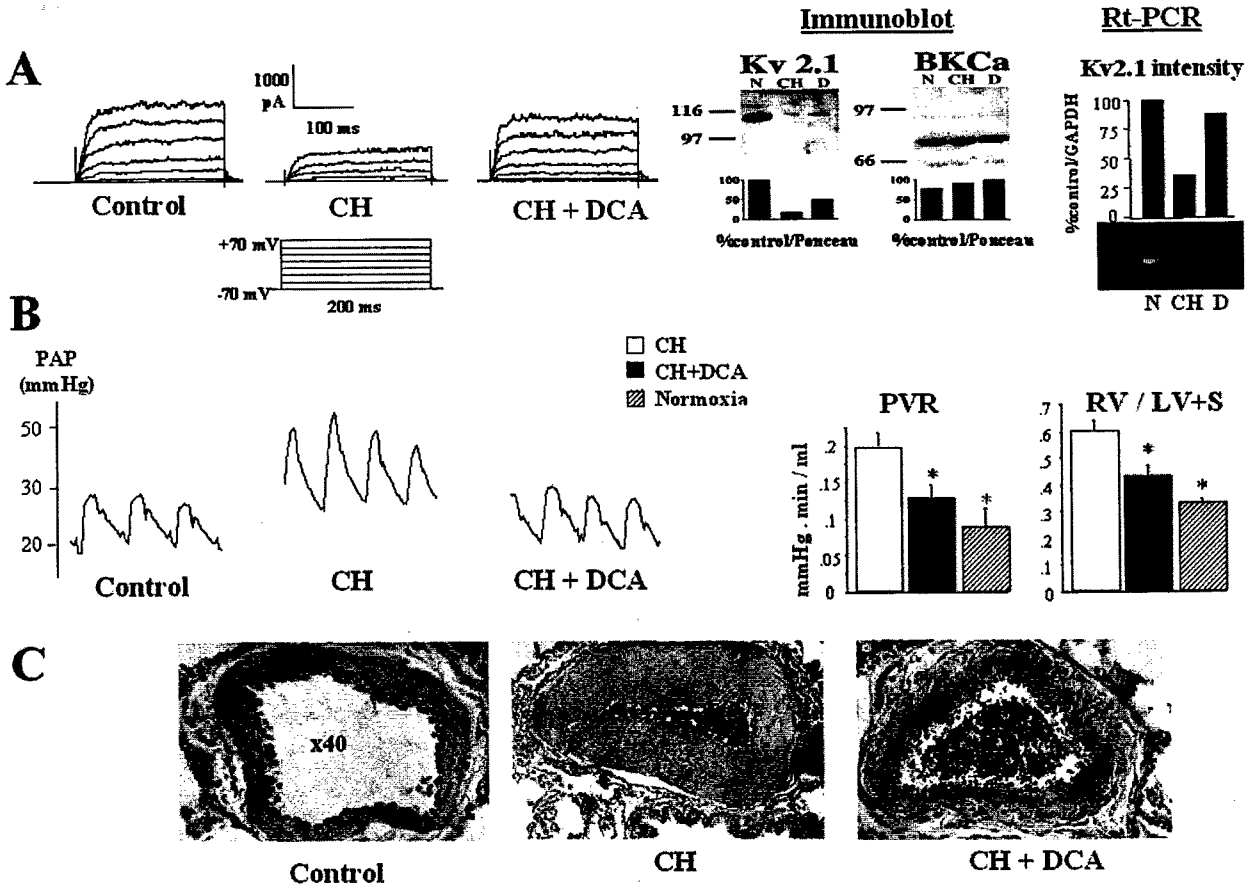

Figure 10. A. Chronic hypoxic suppression of K^+ currents in PASMCs from CH-PHT rats is reversed by DCA. This is associated with upregulation of K^+ channel protein and mRNA ex-pression (on right). **B.** DCA lowers PAP in CH-PHT despite ongoing chronic hypoxia. **C.** DCA causes partial regression of vascular remodeling in small Pas in CH-PHT rats. (representative trace on left, mean data on right). *Obtained with permission from* [18].

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Chapter 22

NON-ERYTHROID FUNCTIONS OF ERYTHROPOIETIN

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Abstract: The oxygen-dependent, renal cytokine eythropoietin (Epo) is well known to increase red cell production. Binding of Epo to the Epo receptor (EpoR) represses apoptosis of erythroid progenitor cells, thereby allowing their final maturation. We and others showed that Epo and its receptor are expressed in many other tissues, including brain, spinal cord, retina and testis. The presence of a blood barrier suggests that Epo plays a local role in these organs. Indeed, therapeutically applied or hypoxically induced Epo has been shown to reduce the infarct volume in various stroke animal models, to prevent retinal degeneration, and to ameliorate spinal cord injury. In a study conducted by Ehrenreich and colleagues, stroke patients treated with Epo showed reduced infarct volume, fast neurological recovery and improved clinical outcome. In analogy to its function on erythroid progenitor cells, this neuroprotective effect of Epo might be explained by repression of programmed cell death. Apart from neuroprotection, there is an assumption that Epo present in breast milk has the potential to protect against mother-to-infant transmission of HIV. When using Epo at high doses for longer time periods; however, care has to be taken to control the resulting chronic polycythemia that most probably caused enlarged cerebral infarct volumes in a transgenic mouse model that due to Epo-overexpression reached hematocrit levels of about 0.8. Overall, these data strongly support the notion that Epo will soon find new applications in the clinic.

Key Words: neuroprotection, stroke, retinopathy, spinal cord injury, HIV

EPO AND ITS RECEPTOR ARE EXPRESSED IN THE MAMMALIAN BRAIN

Until recently, Epo gene expression was thought to be restricted to fetal liver and adult kidney (18). Binding of Epo to its receptor present on erythroid progenitor cells was shown to repress programmed cell death, thereby allowing their final maturation (24). However,

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we and others discovered expression of Epo mRNA in other organs including brain, testis and lung (11, 25, 29, 42). In analogy to the kidney, Epo gene expression was regulated in an oxygen-dependent manner as observed in hypoxic monkeys (29) and mice (11). The presence of the blood-brain-barrier (BBB) excluded a systemic erythropoietic function of brain-derived Epo but suggested a local role of Epo in the brain by binding to local EpoR. Of note, Epo and its receptor were both expressed by neurons and astrocytes (1, 2, 29-31, 33, 34, 40). Interestingly, Epo gene expression in kidney and brain is different, suggesting a tissue-specific regulation: while hypoxia-induced expression of renal Epo peaked at 8h despite continuous exposure of mice to reduced oxygenation, cerebral Epo mRNA levels remained elevated for more than 24h (8). This tissue-specific regulation of Epo gene expression might be the result of the differential modulation of the hypoxic-inducible factor-1 (HIF-1), the key regulator of oxygen-dependent genes such as Epo (17). We have recently shown that the HIF-1-α subunit of this heterodimeric transcription factor peaked in the kidney after 1 h of hypoxic exposure followed by a marked decrease within 8 h despite continuous hypoxia. In contrast, HIF-1α level in the brain reached a plateau after 5h of hypoxia that was maintained for at least 24h (41).

EPO PROTECTS AGAINST BRAIN INJURY IN ANIMAL MODELS

Back in 1998, Sasaki and co-workers reported for the first time that Epo protects neurons from ischemic damage *in vivo*. By occlusion of the common carotid arteries, a model of global ischemia, followed by infusion of Epo into the lateral ventricles of gerbils, the authors observed a reduction of lethal ischemic damage of hippocampal CA1 neurons (37). This protection was reversed by infusing soluble EpoR that prevented Epo from binding to the endogenous EpoR in neuronal cells. The neuroprotective effect of intraventricular injected Epo was confirmed by further studies using rodent models with permanent occlusion of the middle cerebral artery (2, 3, 5, 36), reviewed in (27, 38). Of clinical interest was the fact that intraperitoneally given Epo exerted its neuroprotective function even when applied 6 h after middle cerebral artery occlusion in mice (3).

What are the mechanisms leading to Epo-dependent neuroprotection? Evidence accumulates that, by analogy to the situation during maturation of erythroid progenitor cells to erythrocytes (24), Epo might directly reduce cerebral apoptosis in the ischemic brain, most probably by activating anit-apoptotic genes such as bcl-2 and bcl-xL, or by inhibiting expression of apoptotic genes such as caspases (39). Moreover, Epo has been reported to repress exocytosis of glutamate thereby preventing excitoxic neuronal death (22). Further putative mechanisms are reviewed in Marti and Bernaudin (26).

DOES EPO CROSS THE BLOOD-BRAIN BARRIER (BBB)?

Originally, we and others did not observe any correlation between plasma Epo levels and Epo concentration in the cerebrospinal fluid obtained from patients with an intact BBB (28), even after intravenous injections of 6,000 IU Epo (4). In patients suffering from brain trauma, however, we observed a correlation of Epo levels with the severity of BBB dys-

function (28). These data suggest that Epo does not cross an intact BBB. Cerami and co-workers, however, recently challenged this observation by reporting an active translocation of intravenously applied Epo across the BBB of mice most probably via the EpoR present in brain capillaries. Of note, mice were given high does of recombinant human Epo (5,000 IU/kg body weight). Recently, these authors found that peripherally applied rhEpo into rats peaks in the cerebrospinal fluid after 3.5 h and at about 1% of the peripheral concentration (7). Most probably, Epo enters the brain either upon breakdown of the BBB and/or when it is applied systemically at high doses.

EPO THERAPY FOR ACUTE STROKE IN HUMANS IS BENEFICIAL

Encouraged by their observation that both Epo and EpoR expression is modulated by ischemia in human brain, Ehrenreich and Siren started the first clinical trial using Epo on patients suffering from acute stroke (12). Inclusion criteria of patients were a maximal age of 80, ischemic stroke within the middle cerebral artery territory and symptom onset less than 8 h before administration of the drug. A total of 100,000 IU rhEpo was infused once daily for the first 3 days after stroke. Epo levels in serum and cerebrospinal fluid increased 500 and 60-100 fold, respectively, thereby implying that intravenously applied Epo crosses the (damaged) BBB. Importantly, the authors reported a strong trend for reduction in infarct size in rhEpo-treated patients. This reduction was associated with markedly enhanced neurological recovery and improved clinical outcome as determined one month after stroke. The fact that no side effects of Epo therapy were identified makes the therapeutical use of Epo in ischemia-related neuronal injuries (or even degenerative diseases) very promising for the very near future.

EPO PROTECTS THE HYPOXIC/ISCHEMIC RETINA

Very recently we showed that hypoxic exposure of mice (6% O_2, 6 h) protected the retina from experimentally light-induced retinal degeneration (15). Hypoxic preconditioning induced stabilization of HIF-1 that in turn induced retinal Epo gene expression. The EpoR is required for Epo signalling localized to photoreceptor cells. The protective effect of hypoxic preconditioning was mimicked by intrapertioneal application of rhEpo (5,000 IU/kg body weight) that obviously crosses the blood-retina barrier and prevents apoptosis even when injected therapeutically after exposure to light. Similarly, using a rat model of transient global retinal ischemia induced by increasing intraocular pressure, Junk and co-workers observed that intraperitoneally administered rhEpo reduced morphological and functional damage of the retina (19). Tentatively, application of Epo may be beneficial for the treatment of different forms of retinal disease including acute glaucoma and retinal vascular occlusion, diabetic retinopathy and hypertensive vascular disease.

EPO PROTECTS AGAINST SPINAL CORD ISCHEMIA AND TRAUMA

Spinal cord injury patients are often young, and most survivors face permanent disability. Administration of high-doses of glucocorticoid is the only therapeutical treatment known so far. Because expression of the EpoR was detected in human spinal cord sections, Cerami and co-workers tested in a transient global spinal ischemia model in rabbits, whether Epo crosses the blood-spinal cord barrier to protect ischemic motor neurons from cell death. Indeed, intravenously applied rhEpo prevented motor neuron apoptosis and neurological disability in rabbits in which the abdominal aorta was transiently occluded (6). In keeping with this, the authors reported that acute administration of Epo improves the outcome and accelerates recovery of rats suffering from experimentally induced spinal cord trauma (14). Using two different models of spinal cord injury, one mimicking a brief crush injury (moderate mechanical compression) and the other representing a traumatic contusion (severe mechanical compression), the authors showed that systemically applied rhEpo markedly improved neurological recovery. Does this effect have an impact on patients suffering from spinal cord injury? Despite being difficult and sometimes misleading to translate results from rats to human, it is tempting to mention that an analogous improvement in patients would possibly mean a transition from leg paralysis to restored ambulation with a coordinated gait (13).

DOES EPO MODULATE VENTILATORY RESPONSE TO HYPOXIA?

Considering that Epo and its receptor are present in the brain, we speculated that this cytokine might play a role in the modulation of the ventilatory acclimatization to hypoxic exposure. To test this notion, we made use of our erythrocytotic transgenic mouse model that overexpresses rhEpo preferentially in brain and, to a lesser extent, in lung leading to a 12-fold increase in Epo plasma level (35, 44). Minute ventilation and hypoxic ventilatory response at 6% O_2 were assessed by whole body plethysmography. In a preliminary set of experiments we observed that, when compared to the wildtype control mice, the Epo-overexpressing mice broadly increase respiratory frequency but markedly decrease tidal volume (unpublished observations). Thus it would appear that Epo may have a role in ventilatory acclimatization to hypoxia conditions. Further experiments are now being performed to study whether Epo modulates the ventilatory response during acclimatization to severe hypoxia.

DOES BREASTMILK-DERIVED EPO PROTECT AGAINST MOTHER-TO-INFANT HIV TRANSMISSION?

Up to 1 million HIV-positive children today were infected through breastfeeding, but interestingly, only 15% of the babies from HIV-positive mothers are infected. So how do 85% of the babies escape infection? Miller and co-workers recently presented the hypothe-

sis that Epo present in human milk might protect against HIV infection of babies from HIV mothers (32). Their hypothesis is based on the fact that human milk contains Epo (23) and that EpoR mRNA is found in mammary epithelia (21) as well as in postnatal enterocytes (20). Thus, the authors speculate that Epo in human milk might protect against HIV transmission by either maintaining mammary epithelium integrity (thereby reducing viral load in milk) and/or by maintaining intestinal epithelial integrity in breastfed neonates (thereby preventing ingested milk-derived virus being infective) (32). Needless to mention that this hypothesis is testable by administration of rhEpo to HIV-positive mothers and/or breastfed neonates. If correct this would be a very timely and pertinent therapeutic function for Epo in prevention of spread of HIV.

EPO-INDUCED ERYTHROCYTOSIS

When administering Epo to patients at high doses during longer periods of time, one has to keep in mind that prolonged erythrocytosis might influence the protective effect of Epo. For example, there is evidence in humans that the size of cerebral infarct upon carotid occlusion correlates directly with the hematocrit level (16). We have similar evidence from our transgenic mouse model. In a recent study using our erythocytotic mice overexpressing rhEpo in brain and lung, we reported a deteriorated outcome after stroke (44). We assume that this negative effect is due to the excessive erythrocytosis influencing blood viscosity. In contrast, another transgenic mouse line overexpressing rhEpo exclusively in the brain showed a strong tendency to reduce infarct size after stroke. Apart from this, long-term Epo-induced erythrocytosis might result in multiple organ degeneration. Guided by the reduced life expectancy found in our erythrocytotic transgenic mice overexpressing Epo (43) we analysed 5 to 6 month old transgenic animals. Preliminary analysis revealed severe multiple degenerative processes in skeletal muscle and renal glomeruli (unpublished observations). This further highlights the possible detrimental effects of prolonged high dose Epo usage.

FUTURE DIRECTIONS: NON-ERYTHROPOIETIC EPO?

As mentioned above, the erythropoietic function of Epo might disturb the (neuro)protective one. Thus, one might envision generation of Epo-like drugs that selectively promote neuroprotective rather than erythroid effects. This would require that renal Epo is not identical to its cerebral counterpart or that the erythroid EpoR is distinct from the neuronal one. There are some hints: brain-derived Epo (33 kDa) is smaller in size compared to renal Epo (35 kDa) and might represent different post-translational modifications (31). Moreover, unlike erythroid cells with efficient splicing of EpoR transcripts to its mature form, brain EpoR transcripts are inefficiently or alternately processed (9). Finally, small peptides mimicking the erythropoietic function of Epo (45) also exert a neuroprotective effect *in vitro* (10). These points may enable the development of novel Epo protective or erythrocytotic drugs.

In summary, the experimental data described in this short review strongly supports the notion that Epo has a variety of potential uses that are not only restricted to erythropoiesis

and central nervous system. Considering its long lasting safety profile, we are convinced that current and upcoming clinical trials will soon confirm the widespread therapeutical use of Epo.

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Chapter 23

PETER HOCHACHKA AND OXYGEN

Kenneth B. Storey

Figure 1. Peter W. Hochachka, by Janis Franklin, UBC Media Group, UBC Science 1997.

Peter William Hochachka O.C., Ph.D., L.L.D, F.R.S.C. passed away on September 16, 2002 after a brilliant life of science adventure and leaving as his legacy a whole new field of science - biochemical adaptation.

Peter was an explorer. An explorer of animals and their environments, an explorer of metabolism and its adaptations, and explorer of ideas and concepts. He loved nothing better than the challenge of a new adventure, be it transporting his lab equipment to the far-flung corners of the Earth or devising new theories about how animals work by weaving ideas, readings and conversations into new insights.

Perhaps no modern scientist so enthusiastically embraced the idea first set out in 1865 by Claude Bernard, but later popularized by August Krogh, that stated *"There are also experiments in which it is proper to choose certain animals which offer favorable anatomic arrangements or special susceptibility to certain influences. This is so important that the solution to a physiological or pathological problem often depends solely on the appropriate choice of the animal for the experiment so as to make the result clear and searching."* Peter took this to heart and sought to study the "outer limits" of biochemistry -- animal life that stretched the limits of fastest, deepest, highest, longest! He followed in the steps of the great 20th century comparative physiologists (Krogh, Scholan-

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

der, Prosser, Schmidt-Neilen) and moved the focus down to the intracellular level to study the organization and regulation of energy metabolism. He studied high speed swimming in tuna and squid, high speed flight in hummingbirds. He explored deep diving in seals and the effects of high pressure on the enzymes of abyssal fish. He went to the Andes and the Himalayas to study human hypoxia tolerance at high altitudes. He identified the biochemical adaptations that allow turtles, goldfish and bivalves to live for weeks or months without oxygen. Comparative biochemistry of all types will bear the mark of the "founder effect" of Peter Hochachka for generations to come.

Many of Peter's interests and adventures centered around oxygen. Although his career began with an interest in the biochemical responses to temperature and pressure as environmental stressors, his scientific excursions into how animals deal with variation in oxygen availability soon became the core of his work (Figure 2).

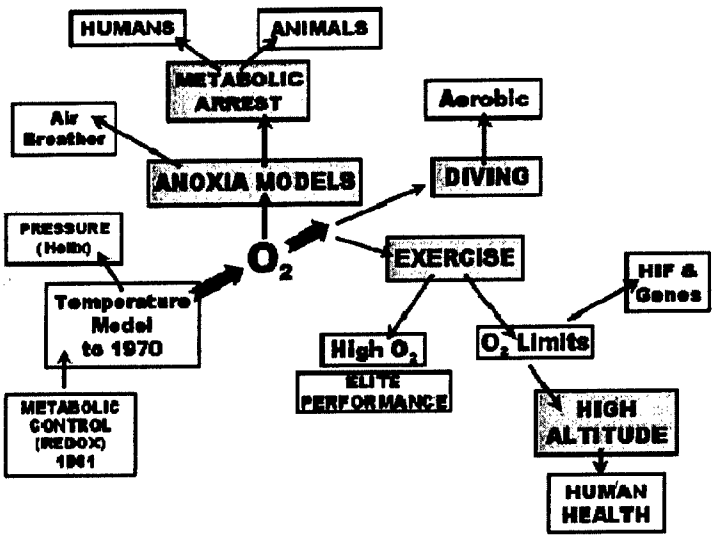

Figure 2. Oxygen was Peter's great scientific love affair - how aerobic metabolism was regulated, how organisms adjusted to hypoxia, how facultative anaerobiosis ensures survival when oxygen is depleted. His ideas ranged over dozens of metabolic problems and animal systems and he attacked problems at multiple levels from whole animal physiology studies in the field, through studies with isolated organs, cells and organelles, and down to purified enzymes and metabolite measurements.

His insights brought integration to a vast field of comparative and medical research on hypoxia/anoxia tolerance and inspired labs around the world to take up his pioneering ideas and study the details of many specific systems. Within the Hypoxia Society, Peter may be best known for his work on hypoxia tolerance in humans and other mammals. This includes nearly 20 years of studies on the biochemistry and physiology of diving in the Antarctic Weddell seal in collaboration with Warren Zapol's group at Harvard as well as ground-breaking analyses of high altitude adaptation in the Quechua people of the Andes

and the Sherpa of the Himalayas. However, many other scientists associate Peter and oxygen with muscle exercise metabolism because another large segment of his career focused on muscle energetics and how high energy outputs were achieved during running, flying, and swimming. Still others, myself included, intersected with Peter through our interests in anaerobic metabolism -- how organisms survive without oxygen. Peter's lab played a central role in the 1970's in eludicating the pathways of anaerobic metabolism in marine molluscs, including discovering and defining the roles of a whole new group of enzymes, the opine dehydrogenases. His lab also produced initial evidence of the importance of reversible protein phosphorylation in metabolic suppression during anaerobiosis and this, plus other leads, evolved into another major focus in his research, the regulation of metabolic rate depression. Studies with turtles, goldfish, and oysters combined with insights from diving mammals and other systems fueled his synthesis of ideas in two books "*Living without Oxygen*" (1980) and "*Metabolic Arrest and the Control of Biological Time*" (1987; with Michael Guppy).

Peter Hochachka was born in Bordenave, Alberta on March 9, 1937. He received his B.Sc. from the University of Alberta, an M.Sc. from Dalhousie University, and then his Ph.D. from Duke University where he also undertook postdoctoral studies. He joined the faculty of the University of British Columbia in 1966 and remained there throughout his career. The excellence of Peter's research is testified to by the many honors that he received including a Guggenheim Fellowship (1977), a Queen Elizabeth II Senior Fellowship (1983), the Killam Research Prize (1978/1988), the Science Council of Canada Gold Medal (1987), the Canada Council/Killam Memorial Prize (1993), the NSERC Gerhard Herzberg Gold Medal for Science and Engineering (1995), the Fry Medal of the Canadian Society of Zoologists (1995), an Honorary Doctorate from St. Francis Xavier University (1998), the Order of Canada (1999) and, posthumously, the Queen's Golden Jubilee Medal. He was an elected fellow of the Royal Society of Canada and an active member of several scientific societies. In recent years Peter took over the editorship of the journal Comparative Biochemistry and Physiology (with Tom Mommsen and Pat Walsh) and took great pride in furthering the development of this journal as a top venue for the publication of studies that explored the limits of biochemical and physiological adaptation in animals and, perhaps more importantly, that provided a key outlet for the research of scientists of many third world nations.

Peter the explorer led or participated in nine research expeditions on the RV *Alpha Helix* to regions as diverse as the Amazon and the Arctic. He also took part in six expeditions to the Antarctic, four to the high Andes, one to the Himalayas and many other research and lecturing trips. In his major expeditions he always joined forces with local scientists to aid their research efforts and he always brought along graduate students to show them the larger world. On these expeditions, Peter was forever adaptable and inquisitive. For example, when we were aboard the *Alpha Helix* in Hawaii and having trouble catching the deep sea fish that we hoped to study, Peter looked over the side of the ship and saw pelagic squid and so began a long series of studies of exercise metabolism in cephalopods that included another *Alpha Helix* expedition, this time to the Philippines to study the elusive *Nautilus*.

Peter was always playing with some new idea. In my graduate student days, he kept the coffee machine beside the Sorvall centrifuge and I have an enduring image of him perched atop the centrifuge, mug in hand, expounding on his latest synthesis. Life events always triggered a drive to learn something new. For example, after the birth of Gail, daughter #2,

all discussion in our graduate seminar class shifted for the next several days to the mechanisms of hypoxia tolerance of the human newborn. A participant at the Banff meeting said to me "You know, I met Peter once in the airport in New Delhi, and he had a great new idea for me about...". Many recollections of Peter revolve around such serendipidous meetings with this always amazing scientist, as he generated and sent out "idea-trails" aimed at key biological questions in any and all aspects of comparative and adaptational sciences. Peter's incessant thirst for knowledge never abated even throughout his illness and, characteristically, the last paper published before his death focused on his own predicament with an analysis of metabolic organization and redox regulation in normal and malignant prostate cells, published with his doctors as co-authors (P.W. Hochachka, J.L. Rupert, L. Goldenberg, M. Gleave, and P. Kozlowski. Going malignant: the hypoxia-cancer connection in the prostate. BioEssays 24: 749-757, 2002).

Peter used what I call "Synthetic Intuition" as his great engine of discovery (Figure 3). He read and understood huge tracts of the scientific literature from ecology through physiology through biochemistry, molecular biology and genetics. He assembled all this information and acted as an 'idea lens' to focus all of this input and design the conceptual framework for deriving the biochemical answers to eco-physiological problems. By culling the thousands of ideas generated by this synthesis into those that could be approached scientifically in the lab or in the field, Peter virtually created the new field of biochemical adaptation. His writings were a revelation of general principles, coupled to practical advice, on how to advance his fields of interest. His entry into an entire new field often started, not with a data-filled research paper, but with a review article that provided a state-of-the art synthesis of the field, sorted to identify the key problems and cogently sketching what should be done to answer these. One such idea-filled article in Science in 1971 "Invertebrate facultative anaerobiosis" helped to draw me into his lab whereas another Science article that I co-authored with him in 1974 "Metabolic consequences of diving in animals and man" set out his new ideas about diving mammals (although at that time we had only one dolphin from the Vancouver Aquarium to provide our experimental material!!). He could take any complex physiological problem and reduce it to testable questions about metabolic pathways, fuel use and cellular energetics. Typically, these were sketched out metabolic maps that provided balanced overviews of the integration of enzymes, pathways, redox regulation, and ATP use. Ideas and approaches suffused his writings, spinning off literally hundreds of studies that could be pursued by labs around the world in every field that his mind touched. He liked nothing better than to regale anyone who would listen with his latest syntheses, refining and playing with the ideas until out would pop another thought-provoking article that was grabbed up by Science or Nature or PNAS.

The environment of Peter's lab was clearly responsible for the success of the many researchers, postdoctoral fellows and the 42 graduate students that passed through it. Peter viewed graduate studies with a "sink or swim" attitude but in a lab where students were bombarded daily with the latest advances gleaned from journals and conferences, with anecdotes from world-wide adventures, and with opportunities to work on just about any animal that peaked their interest, there were ample opportunities to swim. He launched wave after wave of young Canadian students onto the world stage, many of them now leading Figures in the next generation of comparative biochemistry. His was a made-in-Canada, funded-in-Canada enterprise that encouraged students like myself to try new ideas, to attempt difficult projects, and to not fear failure in the conventional sense. His abiding vision

was that experiments, done as well as possible, would always yield the next generation of insights - even if the actual data were revised by future researchers.

Peter had an amazing publishing career. He is by far the most cited scientist in the field of comparative and adaptational biochemistry and, since his first paper in 1962, has published over 400 papers written with more than 200 co-authors. His papers stand out not just for their strong data but also because of his great skill as a storyteller who was able to distill for the reader the key elements of the data and weave them together with an amazingly broad knowledge of medical and biological physiology and biochemistry to derive broad principles of biochemical adaptation. He loved to write thought-provoking articles that challenged readers to follow his new lines of thought and find the applications or the holes.

Figure 3. Synthetic intuition at work, perhaps best described as "Something Old, Something New, Something Borrowed, Some Glue". Peter was a master at drawing together information and ideas from multiple sources, cutting through the overlying confusion of details, and revealing the unifying principles of metabolic regulation underneath. Photo by Dr. Brian Murphy, taken at McMurdo Station, Antarctica, used with permission.

More than anything else, his 1973 book "Strategies of Biochemical Adaptation" (written with George Somero) and published at age 34, established Peter as the major creative force behind the rapidly emerging new field of comparative biochemistry. Its visionary approach brought together ideas from many different fields (physiology, metabolic regulation, enzymology, protein chemistry) and showed how multiple environmental parameters (low oxy-

gen, high pressure, temperature change, osmotic challenge) impacted on metabolism and macromolecules. In the book Peter assembled for the reader a vision of the principles of biochemical design and the options that are available to organisms for crafting their macromolecules and reworking their regulatory mechanisms in order to adapt and flourish in the face of environmental challenges. There is no doubt that that book inspired the research of hundreds of scientists worldwide, myself included, as did the 1984 update "Biochemical Adaptation" and as will the 2002 finale "Biochemical Adaptation: Mechanism and Process in Physiological Evolution" for the next generation of young scientists.

Peter roared through life with immense enthusiasm and a profound love of science. His lectures were spell-binding, his writing a delight and no one could spin a better yarn. He had no use for "knuckle-draggers" and "stamp-collectors" in science, often described new revelations with the phrase "reptilian scales fell from my eyes" and we all learned quickly that "very interesting, very interesting" had quite the opposite meaning. You will never find a better scientific life than Peter crafted. He will be sorely missed by family, friends, students, colleagues and audiences around the world. Goodbye, Pack Leader.

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Chapter 24

PROPOSAL FOR SCORING SEVERITY IN CHRONIC MOUNTAIN SICKNESS (CMS)

Background and Conclusions of the CMS Working Group

Fabiola León-Velarde, Rosann G. McCullough, Robert E. McCullough and John T. Reeves for the CMS Consensus Working Group

INTRODUCTION

Living above 2500 m are millions of persons who are at risk for Chronic Mountain Sickness (CMS), a disorder of excessive red cell and hemoglobin production, which threatens health and even life itself. The CMS Working Group was founded in Matsumoto, Japan and meets regularly at international meetings when a quorum of interested scientists is present. The overall goals of the CMS Working Group are to lay the foundation for better understanding of the description, pathogenesis and treatment of CMS by developing consensus among interested scientists from around the world. Toward that end this chapter presents the latest efforts of the CMS Consensus Working Group.

During the 13th International Hypoxia Symposia high altitude experts representing eight countries met to formalize a scoring system for CMS. Recommended was a scoring system which was a modification of the prior work by Dr. León-Velarde and Dr. Arregui, which was adapted together with C.C. Monge in Spanish. In this chapter is presented first the English translation of "La desadaptación a la vida en las grandes alturas" by R.G. and R.E. McCullough, then the Spanish article is presented, and this is followed by the minutes, including the proposed scoring system, of the Working Group meeting.

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
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THE MALADAPTATION TO LIFE AT HIGH ALTITUDES

Translation: La desadaptación a la vida en las grandes alturas

Translators: Rosann G. McCullough, Robert E. McCullough

(reprinted with permission from León-Velarde, F. and A. Arregui. La desadaptación a la vida en las grandes alturas. Tomo 85. Travaux del Institut Francais d'Etudes Andines (IFEA). Editores, IFEA/ Universidad Cayetano Heredia. Lima, Perú, 1994, IFEA/UPCH).

"...physiological adaptations to altitude are closely linked with processes of maladaptation, and because adaptation and maladaptation are separated by the most subtle of boundaries, it is frequently impossible to say where health ends and where sickness begins..."

-Carlos Monge Medrano, 1928.

In 1925, Carlos Monge Medrano presented to the National Academy of Medicine a communication entitled "Concerning a case of Vaquez' syndrome" (Monge 1925) in which there was a clear association between altitude and the natural erythremia (polycythemia) of the illness. Monge said, "We suspect the existence of Vaquez' syndrome in places situated at 5,000 meters, provided that there is no cause other than the altitude which would abnormally stimulate the bone marrow..." (Monge 1925). In 1928 he described by the name "Sickness of the Andes" the loss of acclimatization or the inability of some individuals (natives or residents) to acclimatize to life at considerable altitudes (Monge 1928). This syndrome, now called Monge's disease or Chronic Mountain Sickness (CMS), has been described over the years in various countries with mountainous regions (Hecht 1958; Ergueta 1971; Kryger 1978a and 1978b; Wu 1987; Pei 1989). It presents in the early stages with symptoms such as migraine, dizziness, restlessness, somnolence or insomnia, fatigue, muscle and joint pain, loss of appetite, lack of mental concentration and alterations of memory, localized cyanosis, burning in the palms of the hands and soles of the feet and dilatation of the veins, among other signs and symptoms. An elevated hemoglobin (polycythemia), arterial hypoxemia and hypercapnia accompany the clinical picture (Monge 1966; Ergueta 1971; Winslow 1987), the hemoglobin concentrations being greater than expected for the altitude of residence. In the early stages of the illness, the signs and symptoms described predominate, while in the final stages, cardiopulmonary signs and symptoms, such as cor pulmonale, predominate (Peñalosa 1971). In the absence of pulmonary illness, hypoventilation is accepted as the primary cause of CMS (Hurtado 1942, 1960; Erguets 1971). The frequency of CMS is in direct relation to the altitude (Cosio 1965, 1968), however once the illness occurs, it progresses with the same severity at all altitudes.

According to Monge, the elevated hemoglobin concentration is the most prevalent sign of the illness: "The clinical history which accompanies the illness is perfectly compatible with respect to a characteristic erythremia" (Monge 1925). He also says, "There are afflicted persons with complex syndromes, there are afflicted persons with disassociated

syndromes, but in each case there is erythremia as a basic element of the illness. We have called this 'polycythemia of altitude'."

Given that the principal manifestation of the disease is the excessive erythrocytosis, it is necessary to create diagnostic criteria for CMS based upon an easily obtained score which would integrate the main signs and symptoms of CMS and which could be shown to be associated with the excessive erythrocytosis.

STUDY DESIGN

The study was carried out in the form of a questionnaire with an *a posteriori* medical examination of respondents selected from an urban population of 72,000 inhabitants with a sufficient level of education to facilitate communication among the physicians, the questionnaire surveyors and the target population. The urban life style of this population is comparable to those of other epidemiological studies in Andean populations. A sample size was determined which would yield confidence intervals of 95% for all of the estimates of interest, and which would permit the comparison of certain variables between two groups (miner vs. non-miners; limited to 1/3 of the total sample).

SAMPLING TECHNIQUES

The sampling scheme was constructed on the basis of maps of the district of Cerro de Pasco, available from the district municipality. The maps provided details down to the level of the residential block. The random sampling took place in three districts which were representative of the city of Cerro de Pasco: Chaupimarca, San Juan and Paragsha.

In order to obtain a preliminary idea of the residential areas, we examined the districts selected in order to exclude commercial zones and non-residential areas (university complexes, schools and sports fields). The zones which remained were divided into groups by blocks, each group containing different neighborhoods. To satisfy the sampling requirements, the groups selected were sorted and assigned numbers.

QUESTIONNAIRE

With the aim of observing the largest number of signs, symptoms and/or risk factors associated with long-term exposure to chronic hypoxia, questions were designed to have high sensitivity. Subsequently, cases which indicated certain medical problems would be given a clinical examination including the measurement of hemoglobin concentration.

Criteria for Inclusion

Age: The study focused on persons aged 20 years or older since the effects of maladaptation to altitude increase with age (Arregui 1990; León-Velarde 1993). Length of residence in Cerro de Pasco: Persons who had lived in the city for 10 years or more and

who had not lived at low altitude for more than three months during the previous year were considered to be "resident."

Questionnaire Surveyors

The surveyors (n=7) were recruited from students in their last year of the program for infirmary assistants of the Daniel Alcidses Carrion National University of Cerro de Pasco, Faculty of Health Sciences. The two field supervisors of the surveyors were from the Department of Physiological Sciences of the Cayetano Heredia University of Peru. The team of investigators (principals, associates and assistants) consisted of eight physicians (three internists and five neurologists), one physiologist, one sociologist and one epidemiologist. The surveyors, the field supervisors and the investigators participated in training with the objective of clarifying for the surveyors both their job and the objective of each of the parts of the questionnaire. The aim was to create a well-integrated team to carry out the objectives of the field work. The surveyors received instruction from the epidemiologist in order to achieve random sampling using random number tables. The group was divided into three teams, each with an assigned district as its responsibility. The questionnaire was administered by the surveyors. If the respondent answered affirmatively to a question concerning a respiratory or neurological symptom, that person was examined by the physician of the team.

Design

The questionnaire, administered to 473 male subjects, was divided into four sections, each one designed to obtain a profile in accordance with the objectives of the project, *i.e.*, respiratory, neurological, physiological and sociological aspects. Each section was revised by specialists and the overall coherence of the questionnaire was coordinated by the epidemiologist and the project administrator.

The questionnaire contained data such as socio-demographic indices, migration history, perceptions of health, consumer habits, acute morbidity, vital functions and laboratory analyses, respiratory and neurological diagnoses, perceptions concerning jobs, signs and symptoms of depression and signs and symptoms of CMS. It is this last part of the questionnaire with which we are concerned in this report.

The signs and symptoms most prevalent in CMS include physical and mental fatigue, depression, dizziness, headache, stinging or burning sensations in the hands or feet, muscular or bone pain, shortness of breath, the presence of cyanosis, dilation of the veins and tinnitus (Monge 1928; Monge 1966; Monge MC personal communication). It was on the basis of these signs and symptoms that we created the following items of the questionnaire:

1. Do you suffer from headaches?
   (1) Never (2) Occasionally (3) Frequently

2. Do you have muscle or bone pain?
   (1) Never (2) Occasionally (3) Frequently

3. Do you have ringing in your ears?
(1) Never (2) Occasionally (3) Frequently

4. Do you have dizziness?
(1) Never (2) Occasionally (3) Frequently

5. Do you have burning or stinging sensations on the soles of your feet or the palms of your hands?
(1) Never (2) Occasionally (3) Frequently

6. Do you have difficulty in breathing? Do you feel short of breath?
(1) Never (2) Occasionally (3) Frequently

7. Do you have difficulty sleeping well?
(1) Never (2) Occasionally (3) Frequently

8. Do you have a good appetite?
(1) Frequently (2) Occasionally (3) Never

9. Do your face or your hands appear blue or purple?
(1) Slightly (2) Moderately (3) Severely

10. Do you have enlargement (dilation) of the veins of your hands or your feet?
(1) Slightly (2) Moderately (3) Severely

11. Are you physically tired?
(1) Slightly (2) Moderately (3) Severely

12. Are you mentally tired?
(1) Slightly (2) Moderately (3) Severely

In the first stage, the CMS score was calculated from the responses obrained. The minimal score possible was 10 and the maximum 28. In a second phase, the score was evaluated as follows:

0	Negative response
1	Two or fewer episodes per month or "moderate"
2	More than two episodes per month or "severe"

The scores given to the lines are 1 point for questions 2, 4, 8, 11 and 12; 2 points for questions 5, 6, 7, 9 and 10; and 3 points for questions 1 and 3. In addition, 3 points are given for a hemoglobin concentration greater than two times the standard deviation for the altitude of residence (>21.3 g/dl for Cerro de Pasco) and/or for an oxygen saturation less than two times the standard deviation for the altitude of residence (<82% for Cerro de Pasco). From this composite score the categories are assigned as follows:

<12	Healthy
12-18	Slight CMS
19-24	Moderate CMS
>24	Severe CMS

The Predictive Value of the CMS Score

Epidemiological procedures were used to calculate predictive values, sensitivity and specificity with the goal of validating the questions concerning the symptomatology associated with CMS and also the CMS score generated. As a standard for the diagnosis of illness we used the hemoglobin concentration. The threshold used to consider the hemoglobin as being elevated was the mean value for the young (20-30 yrs), adult, male population plus two standard deviations to the right (Hb=21.3 g/dl in Cerro de Pasco) (Arregui 1990; León-Velarde 1993). Normal and elevated hemoglobin were assigned vales of "-" and "+" respectively.

The questions whose responses are considered most important are evaluated in the following manner:

		Hemoglobin (g/dl) >21.3	<21.3	
		+	-	
Question	Yes (+)	a	b	a + b
or test	No (-)	c	d	c + d
		a + c	b + d	a+b+c+d

Calculations:
Sensitivity: a/(a+c)
Specificity: d/(b+d)
Predictive value:
Positive a/(a+b)
Negative d/(c+d)
Presence of illness: (a+c)/(a+b+c+d)

In Table 1 we have summarized values for sensitivity, specificity, the predictive value, the presence of illness and the presence of excessive hemoglobin or erythrocytosis for some of the questions which compose the CMS score questionnaire and the CMS score calculated.

CONCLUSIONS

The CMS score described herein has high specificity, a high negative predictive value and a significant association with the presence of elevated hemoglobin (Hb>21.3 g/dl, an "odds ratio" of 0.91). We have defined CMS in a population at altitude (>1,600 m) where, after administering questions 1 through 10 of the questionnaire, we obtained the following scores:

<12	Healthy
12-18	Slight CMS
19-24	Moderate CMS
>24	Severe CMS

At 4,300 m in Cerro de Pasco the prevalence of CMS in the adult population is 15% (Monge, 1992).

Table 1. Evaluation of some of the questions which compose the components of the CMS score and of the CMS score when compared with Hb>21.3 g/dl (standard).

	Sens	Spec	Predictive value		Presence of illness	% Hb>21.3	
			+	-		No	Yes
Symptom:							
Physically tired:							
Occasionally	0.36	0.55	0.12	0.84	0.14	16	12
Frequently	0.30	0.74	0.18	0.84	0.17	16	18
Mentally tired:							
Occasionally	0.45	0.41	0.10	0.83	0.13	17	10
Frequently	0.38	0.74	0.25	0.83	0.19	17	25*
Dizziness:							
Frequently	0.35	0.58	0.14	0.82	0.16	18	14
Shortness of breath:							
Frequently	0.11	0.94	0.23	0.86	0.15	14	23*
Cyanosis							
Occasionally	0.59	0.64	0.19	0.91	0.13	9	19*
Frequently	0.40	0.88	0.31	0.91	0.12	9	31*
Headache							
Type: tension	0.25	0.79	0.11	0.90	0.10	10	11
Type: migraine	0.56	0.66	0.21	0.90	0.14	10	21*
CMS score > 21	0.17	0.91	0.25	0.86	0.15	14	25**

* $p<0.005$ when compared with those who answered "No" ** $p<0.005$ when compared with those who had a CMS score >21

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ORIGINAL ARTICLE: LA DESADAPTACION A LA VIDA EN LAS GRANDES ALTURAS

The maladaptation to life at high altitudes

Fabiola León-Velarde and Carlos Monge C.

...es fácil darse cuenta de que el estudio de los mecanismos fisiológicos de adaptación a esas alturas se vincula estrechamente con los procesos fisiológicos de desadaptación, ya que unos y otros apenas si están separados por linderos tan sutiles que es imposible con frecuencia decir adonde concluye el estado de salud y comienza el de enfermedad....

Carlos Monge Medrano, 1928.

En el año 1925 Carlos Monge M. presentó a la Academia Nacional de Medicina una comunicación "Sobre un caso de Enfermedad de Vaquez (Síndrome eritrémico de altura)" en la que se aprecia la asociación clara entre altura y la naturaleza eritrémica del enfermo. Dice Monge M.: ..."De otro lado era de sospechar la existencia de casos de la enfermedad de Vaquez en lugares situados a 5,000 m, dado que ninguna causa patógena mas aparente que la altura para excitar anormalmente la médula osea..." (Monge M., 1925). En 1928 describió con el nombre de Enfermedad de los Andes a la pérdida de aclimatación o a la incapacidad de algunos individuos (nativos o residentes) de aclimatarse en forma integral a la vida a alturas considerables (Monge M. y col., 1928). Este síndrome, llamado ahora En-

fermedad de Monge o Mal de Montaña Crónico (MMC), ha sido descrito, con el correr de los años, en diversos países con regiones montañosas (Hecht y McClement, 1958; Ergueta y col., 1971; Kryger *et al.*, 1978a y b; Wu y col., 1987; Pei y col., 1989). Se presenta con manifestaciones como cefaleas, mareos, nerviosidad, somnolencia o insomnio, fatiga, dolores en los músculos y articulaciones, inapetencia, falta de concentración mental y alteraciones de la memoria, cianosis localizada, quemazón en las palmas de las manos y plantas de los pies, dilatación de las venas, entre otros síntomas y signos. Una elevada cifra de hemoglobina, hipoxemia e hipercapnia arterial acompañan al cuadro clínico (Monge M. y Monge C, 1966; Ergueta y col., 1971; Winslow y Monge C., 1987), estando los valores de hemoglobina por encima de aquellos esperados para la altura de residencia. En las etapas tempranas de la enfermedad predominan los signos y síntomas descritos mientras que en las finales predominan los cardiopulmonares como el cor-pulmonale (Peñaloza y col., 1971). En ausencia de enfermedad pulmonar se acepta a la hipoventilación como causa primaria del MMC (Hurtado, 1942; 1960; Ergueta y col., 1971). La frecuencia del MMC esta en relación directa con la altura (Cosio, 1965; 1968), sin embargo, una vez producida la enfermedad, esta evoluciona con igual gravedad en todos los niveles altitudinales.

Segun el propio Monge M., la elevada concentración de hemoglobina es el signo más preponderante de la enfermedad: ..."La historia clínica que acompaño es perfectamente concluyente respecto de la naturaleza eritrémica del enfermo" (Monge-M., 1925). También dice: .."Habrá enfermos con síndromes complejos, habrá enfermos con síndromes disociados, pero siempre en todos ellos se encuentran los síntomas eritrémicos como elementos básicos de la afección. He aquí porque la hemos llamado eritremia de las alturas"....

Dado que el signo principal de la afección es la eritrocitosis excesiva, nos planteamos la necesidad de generar un criterio diagnóstico para el MMC basado en un puntaje de fácil aplicación que integre los principales síntomas y signos del MMC y que se encuentren asociados a la eritrocitosis excesiva.

DISEÑO DEL ESTUDIO

El estudio se realizo en base a una encuesta de tipo transversal (con posterior examen médico de los casos evocados) sobre una población urbana total de 72,000 habitantes con un buen nivel de instrucción que facilitaba la comunicación entre médicos, encuestadoras y población. El modo de vida urbana de esta población la hace comparable a otros estudios epidemiológicos en poblaciones andinas. El tamaño de la muestra se determinó de manera de obtener intervalos de confianza del 95% para todos los estimados de interés, y de permitir la comparación de ciertas variables entre 2 grupos (mineros y no mineros; limite 1/3 de la muestra total)

TECNICAS DE MUESTREO

El marco muestral se construyó en base a mapas del distrito de Cerro de Pasco, disponibles en la Municipalidad del distrito, con detalle hasta a nivel de cuadras. El muestreo al azar se realizó en tres barrios (sectores) representativos de la ciudad de Cerro de Pasco: Chaupimarca, San Juan y Paragsha.

Para obtener una idea preliminar de las viviendas se recorrieron en 2 días los barrios escogidos eliminando zonas comerciales y las no destinadas a viviendas (complejo universitario, escuelas, campos deportivos). Las zonas que calificaban se dividieron en conglomerados por manzanas y cada conglomerado podía contener diferentes barrios. Para cumplir los requisitos de la muestra se sortearon, asignándoles un número, los conglomerados escogidos más los de reemplazo.

ENCUESTA

Con el fin de detectar el mayor número de signos, síntomas y/o factores de riesgo asociados a la exposición permanente a hipoxia crónica, se diseñaron preguntas de alta sensibilidad. Posteriormente, los casos problema evocados en la encuesta serían confirmados por medio del examen clínico y la medida de la concentración de hemoglobina.

Criterios de Inclusión

Edad: el estudio se centró en personas con edad superior o igual a los 20 años ya que los efectos de la desadaptación a la altura se incrementan con la edad (Arregui y col., 1990; León-Velarde *et al.*, 1993). Tiempo de residencia en Cerro de Pasco: Se consideró como residente a las personas que vivían en la ciudad un tiempo no menor de 10 años, y que no hubieran viajado a zonas bajas por periodos mayores de 3 meses en el último año.

Encuestadores

Las encuestadoras (N=7) fueron reclutadas entre alumnas del último año del programa para auxiliares de enfermería de la Universidad Nacional Daniel Alcides Carrión de Cerro de Pasco (Facultad de Ciencias de la Salud). Las supervisoras de campo de las encuestadoras fueron dos asistentes del Departamento de Ciencias Fisiológicas de la Universidad Peruana Cayetano Heredia. El equipo de investigadores (principales, asociados y asistentes) consistió de ocho médicos (tres internistas y cinco neurólogos), una fisióloga, un sociólogo y un epidemiólogo. Encuestadoras, supervisoras de campo e investigadores participaron del entrenamiento con el objeto de aclarar a las encuestadoras, tanto su labor como los objetivos de cada una de las partes de la encuesta. Se buscó que el equipo se encontrara bien integrado para llevar a cabo el trabajo de campo. Las encuestadoras recibieron instrucciones del epidemiólogo sobre como realizar el muestreo al azar con la utilización de las tablas de números aleatorios. El grupo se dividió en 3 equipos, cada uno con un determinado barrio a su cargo. La encuesta fue realizada por las encuestadoras. Si el encuestado contestaba afirmativamente a alguna pregunta respiratoria o neurológica, era examinado por el médico del equipo.

Diseño

La encuesta, aplicada a 473 sujetos de sexo masculino, se dividió en 4 secciones, cada una de ellas dirigida a obtener un perfil del poblador de acuerdo a lo delimitado por los

objetivos del proyecto, i.e., aspectos respiratorios, neurológicos, fisiológicos y sociológicos. Cada sección fue revisada por especialistas y la coherencia global de la encuesta fue corroborada por el epidemiólogo y por el consultor del proyecto.

La encuesta contenía datos sobre, índices socio-demográficos, migración, sobre percepción de salud, hábitos de consumo, morbilidad aguda, funciones vitales y análisis de laboratorio, diagnósticos respiratorios, diagnósticos neurológicos, percepción sobre el trabajo, síntomas y puntaje de depresión y sobre síntomas y signos (puntaje) de Mal de Montaña Crónico. Es de esta última parte de la encuesta de la que nos ocupamos en este artículo.

En base a algunos de los signos y síntomas de mayor prevalencia en el MMC (Monge M. *et al.*, 1928; Monge M. y Monge C., 1966; Monge C., comunicación personal) como: cansancio físico y mental, tristeza o depresión, mareos, dolores de cabeza, ardor o quemazón en las manos o los pies, dolores musculares o articulares, falta de aire al despertar, presencia de cianosis, dilatación de las venas y tinnitus, se hicieron las siguientes preguntas en la encuesta:

1. Sufre ud. de dolores de cabeza?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

2. Siente ud. dolores en los musculos o en los huesos?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

3. Sufre ud. de zumbidos en los oidos?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

4. Siente mareos?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

5. Le queman o arden las plantas de lo pies o las palmas de manos?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

6. Tiene dificultades para respirar bien? Siente que le falta el aire?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

7. Tiene dificultades para dormir bien?
   (1) Nunca (2) Ocasionalmente (3) Frecuentemente

8. Tiene buen apetito
   (1) Frecuentemente (2) Ocasionalmente (3) Nunca

9. La cara o las manos se le han puesto azules o morados?
   (1) Levemente (2) Moderadamente (3) Severamente

10. Tiene dilatadas las venas de las manos o de los pies?
    (1) Levemente (2) Moderadamente (3) Severamente

11. Siente ud sensacion de cansancio fisico?
    (1) Levemente (2) Moderadamente (3) Severamente

12. Siente ud sensacion de cansancio mental?
    (1) Levemente (2) Moderadamente (3) Severamente

Inicialmente, se calculó el puntaje de MMC de acuerdo a las respuestas obtenidas. El puntaje mínimo posible fue de 10 y el máximo de 28. Posteriormente el puntaje se modificó como sigue: (0) = respuesta negativa, (1) = 2 o menos episodios por mes o moderado; (2) = más de 2 episodios por mes o severo. El puntaje dado a cada pregunta fue: 1 punto para las preguntas 2, 4, 8, 11, y 12; 2 puntos para las preguntas 5, 6, 7, 9 y 10; 3 puntos para las preguntas 1 y 3, y para una concentración de hemoglobina mayor a 2 veces la desviación estándar para la altura de residencia (> 21.3 g/dl para Cerro de Pasco) y/o una saturación de oxígeno menor de 2 veces la desviación estandar para la altura de residencia (82%). Con este nuevo puntaje los cortes se hicieron como sigue: puntaje menor de 12, sano; de 12 a 18, MMC leve; de 19 a 24, MMC moderado y mayor de 24 MMC severo.

Valor Predictivo del Puntaje de MMC

Se utilizaron procedimientos epidemiológicos como el cálculo de valores predictivos, sensibilidades y especificidades, con el fin de validar las preguntas sobre la sintomatología asociada al MMC y el puntaje de MMC que ellas generarían. Como estándar para el diagnóstico de la enfermedad se utilizó la concentración de hemoglobina, signo preponderante de ésta. El punto de corte tomado para considerar a la hemoglobina como signo + o - de la enfermedad fue el valor promedio de la población masculina adulta jóven (20-30 años) + 2 desviaciones estándar a la derecha (Hb = 21.3 g/dl) (Arregui *et al.*, 1990; León Velarde *et al.*, 1993).

Las preguntas cuyas respuestas se consideraron mas prevalentes se evaluaron de la siguiente manera:

'Estandar' para el diagnóstico de la enfermedad

		Hemoglobina (g/dl) >21.3	<21.3	
		+	-	
Pregunta	Si (+)	a	b	a + b
o prueba	No (-)	c	d	c + d
		a + c	b + d	a+b+c+d
Sensibilidad:	a/(a+c)			
Especifidad:	d/(b+d)			
Valor predictivo:				
Positivo	a/(a+b)			
Negativo	d/(c+d)			
Prevalencia de enfermedad:		(a+c)/(a+b+c+d)		

En la Tabla 1 se resumen las sensibilidades, especificidades, el valor predictivo, la prevalencia de enfermedad y la prevalencia de hemoglobina excesiva o eritrocitosis excesiva (i.e., Hb > 21.3 g/dl o EE) de algunas de las preguntas que componen el puntaje de MMC y del puntaje de MMC obtenido.

	Sens	Espec	Valor Predictivo		Prev.	% con Hb>21.3	
			+	-		en No	en Si
Sintiomas:							
Fisicalmente cansado:							
Ocasionalmente	0.36	0.55	0.12	0.84	0.14	16	12
Frecuentamente	0.30	0.74	0.18	0.84	0.17	16	18
Mentalmente cansado:							
Ocasionalmente	0.45	0.41	0.10	0.83	0.13	17	10
Frecuentamente	0.38	0.74	0.25	0.83	0.19	17	25*
Mareos:							
Frequently	0.35	0.58	0.14	0.82	0.16	18	14
Falta de aire:							
Frecuentamente	0.11	0.94	0.23	0.86	0.15	14	23*
Tener cianosis:							
Ocasionalmente	0.59	0.64	0.19	0.91	0.13	9	19*
Frecuentamente	0.40	0.88	0.31	0.91	0.12	9	31*
Tener cefalca:							
Type: tension	0.25	0.79	0.11	0.90	0.10	10	11
Type: migraine	0.56	0.66	0.21	0.90	0.14	10	21*
Puntaje MMC > 21	0.17	0.91	0.25	0.86	0.15	14	25**

Tabla 1. Evaluación de algunas preguntas que componen el puntaje de MMC, y del puntaje de MMC, cuando se les compara con Hb > 21.3 g/dl (estándar).

* $p < 0.005$ cuando se compara con prevalencia de Hb > 213 en los que contestan No. **$p < 0.005$ cuando se compara con prevalencia de Hb > 213 en los que tienen pMMC < 21.

CONCLUSIONES

El puntaje de MMC tiene una alta especificidad, un alto valor predictivo negativo y una asociación significativa ($p<0.005$) cuando se le compara con una prevalencia de Hb > 21.3 g/dl, siendo el "odds ratio" de 0.91. Definimos entonces MMC en una población de altura (> de 1,600 m) cuando después de aplicadas las preguntas del 1 al 10 se obtienen los siguientes puntajes: puntaje menor de 12, sano; de 12 a 18, MMC leve; de 19 a 24, MMC moderado y mayor de 24 MMC severo. A 4,300 m de altura la prevalencia de MMC en la población adulta de Cerro de Pasco fue de 15% (Monge *et al.*, 1992).

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MINUTES OF CMS WORKING GROUP 2003

Fabiola León-Velarde, President; John T. Reeves, Secretary,
Chronic Mountain Sickness Working Group

February 21, 2003, 2:00 PM
Banff Center, Banff Canada
Hypoxia Symposium XIII

Present: Fabiola León-Velarde, Chair (Perú); Buddha Basnyhat (Nepal); Luciano Bernardi (Italy); Peter Hackett (USA); Li Ri Ge (China); Marco Maggiorini (Switzerland); John T. Reeves (USA); Jean Paul Richalet (France); Robert Roach (USA); Enrique Vargas (Bolivia).

1. Announcements:
   Dr. León-Velarde informed that the most important health problems for world mountainous populations were presented to the representatives of the "Red de Vigilancia Epidemiológica de la Comunidad Andina" ("Convenio Hipólito Unanue")

by herself and Pr. Gustavo Gonzales, Director of the "Instituto de Investigaciones de la Altura (IIA). The great achievement of this meeting was that representatives of all Andean countries (Bolivia, Colombia, Ecuador, Perú, Venezuela and Chile) agreed to include high altitude diseases as part of the 5-years agenda of the Ministries of Health of the Andean Community held in Lima the 28-29th of November 2002. This is important to facilitate funding for documentation, research, and effective treatment of these diseases and to promote dissemination of information to the public. Thus the working group sees this as an important step toward promotion of health for literally millions of high altitude dwellers.

2. Guidelines for documentation of CMS. In view of the recognition of high altitude diseases as a health problem, it was incumbent on the Consensus Working Group to take the first step to assist the various Ministers of Health in understanding how the working group views criteria for diagnosis of CMS. CMS, also known as Monge's disease or excessive erythropoiesis, occurs in persons who are residents of high altitude, and who have excessively high hemoglobin, hematocrit, and/or red cell values which are causing symptoms or disability. (Other disorders which may occur at, or be facilitated by, high altitude residence will be considered at future meetings.) The group wanted to start with CMS in view of the large amount of work which has already been done in this area.
   a. Exclusion Criteria.
      i. The consensus group considers that a diagnosis of CMS should be made in persons without chronic pulmonary diseases such as pulmonary emphysema, chronic bronchitis, bronchiectasis, cystic fibrosis, lung cancer.
      ii. Males with hemoglobin values less than 18 grams per 100 ml of blood and females with hemoglobin values less than 16 grams per 100 ml of blood are excluded from the diagnosis of CMS.
      iii. Persons living below an altitude of 2500 m are excluded from diagnosis of CMS.
   b. Scoring System for: breathlessness and/or palpitations; sleep disturbance; cyanosis; dilatation of veins; paresthesias:

For each of the above, if absent,	score = 0
For each of the above, if occurrence is once or twice a month;	score = 2
For each of the above, if occurrence is 3 or more a month or severe	score = 4

   c. Scoring System for headache; tinnitus:

For each of the above, if absent,	score = 0
For each of the above, if occurrence is once or twice a month;	score = 3
For each of the above, if occurrence is 3 or more a month or severe	score = 6

   d. Scoring System for blood hemoglobin concentration (gm/100 ml):

Males: greater than 18, but less than 21	score = 0
Males: 21 or greater	score = 6
Females: greater than 16, but less than 19	score = 0
Females: 19 or greater	score = 6

e. Overall Scoring System, which sums b through d, above, and yields a maximal possible score of 38. CMS assessment by score is as follows:

No CMS	overall score = 0 through 9
Mild CMS	overall score = 10 through 16
Moderate CMS	overall score = 17 through 22
Severe CMS	overall score = 23 or higher

3. Translation of chapter from Spanish to English. Dr. León-Velarde and Dr. Arregui have written a chapter in Spanish, which deals with the above guidelines and system. She will send this electronically to Mr. R. E. McCullough and Mrs. R.G. McCullough, who will translate the adapted version of the chapter into English for publication in the Proceedings of the Hypoxia Symposium.

4. Based on the above guidelines, the members of the working group will continue the evaluation of high altitude populations.

Chapter 25

EPIDEMIOLOGICAL MODELING OF ACUTE MOUNTAIN SICKNESS (AMS)

A prospective data collection standard

Richard D. Vann, Neal W. Pollock, Carl F. Pieper, David R. Murdoch, Stephen R. Muza, Michael J. Natoli, and Luke Y. Wang

RATIONALE

Altitude exposure is the cause of acute mountain sickness (AMS), but individual and environmental factors affect AMS susceptibility. As such, standard statistical modeling methods can be used to combine AMS data from dissimilar altitude-time exposures for simultaneous analysis (1). This approach can: (a) support hypothesis testing concerning possible AMS risk factors; (b) aid in ascent planning; and (c) assist in clinical management by providing estimates of individual prognosis. Preliminary work suggested that statistically significant results could be achieved with a population of only several hundred individuals when the AMS incidence was about 50% (1). Complex investigations could require larger populations.

PROPOSAL

Our preliminary work was based on subjects who were partially acclimatized when they entered the study (1). To develop a robust statistical model of AMS, data for subjects who are initially unacclimatized are required with measures of AMS at altitudes of 2,000–5,000 m over a wide range ascent rates. We propose a multi-center approach to developing this database according to the format described below. Prospective data are preferred, but retrospective data that meet the stated requirements are useful. Data may have been obtained during laboratory experiments, field studies, or expeditions. Data should be submitted to Dr. Stephen R. Muza at the U.S. Army Research Institute of Environmental Medicine (USARIEM), Natick, MA where the database will be maintained. Participating investigators will be joint authors on publications that use their data.

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

REQUIRED INFORMATION

The minimum information for each individual in a study population includes: (a) the altitude-time profile of the exposure; (b) a corresponding measure of AMS – Lake Louise Questionnaire (2) or Environmental Symptoms Questionnaire (3) – for each altitude-time datum; and (c) relevant conditions or occurrences (time of day, exercise, thermal state, medication, oxygen, etc.) corresponding to each altitude-time datum; and (d) information regarding demographics, medical and altitude exposure history, medications, and altitude exposure within the previous month. Ideally, one datum should be obtained in the morning. Additional measures (e.g., pulse oximetry values, heart rate, ventilation, etc.) would be welcomed from more extensive investigations. The altitude-time exposure should begin at the individual's resident altitude at a zero time reference and end with recovery from AMS before or upon return to low altitude. (Ultimate recovery is an important descriptor of acclimatization.) All information should be recorded from the zero time reference. A minimum of one altitude-time-AMS datum should be recorded each day. Time should be recorded as decimal days.

DATA CONFIDENTIALITY

Only data collected under the auspices of an active, approved Institutional Review Board (IRB) protocol can be accepted. Documentation of approval must be provided. To ensure individual privacy and compliance with the Health Insurance Portability and Accountability Act (HIPAA is a U.S. requirement), individual identifying information (e.g., name, address, birth date [or age if greater than 90 years], personal identification numbers, etc.) must be deleted from submitted data and replaced by a random code. Linkage between the code and identifying information should be destroyed upon submission. Identification of the data source should be limited to the year and country in which the exposure began and to whether the data were obtained during a laboratory experiment, field study, or expedition.

DATA FORMAT

Data should be stored in a standard database program (Excel, Access, tab delimited text, etc.) with one record per observation for each individual as indicated in the examples of Tables 1-3. Headers should define the nature of the data. Tables 1 and 2 describe the recommended altitude-time formats when AMS is measured by the Lake Louise (2) or ESQ (3) scores, respectively. Medications, oxygen, activity level, temperature, pulse oximetry values, etc. can be added as extra fields to Tables 1 and 2. Table 3 is an example of relevant pre-exposure or baseline information.

Table 1. Recommended data format for studies using Environmental Symptoms Questionnaire (3)

Subject ID	Time	ET	Pb	I feel light-headed	I have a head-ache	I feel sinus pressure	I feel dizzy	I feel faint	My vision is dim	My coord-ination is off	I am short of breath	It is hard to breathe	Etc.
A99546	7:00	-1.138	764	0	0	0	0	0	0	0	0	0	.....
A99546	20:00	-0.597	764	0	0	0	0	0	0	0	0	0	.....
A99546	7:00	-0.138	752	0	0	0	0	0	0	0	0	0	.....
A99546	20:00	0.403	445	0	0	0	0	0	0	0	0	0	.....
A99546	7:00	0.862	445	0	2	0	1	0	0	0	2	0	.....

Time: time of day in 24-hour format
ET: elapsed time since start of ascent
Pb: barometric pressure (mmHg) at time of AMS evaluation

Table 2. Recommended data format for studies using Lake Louise AMS Scoring System (2).

Subject ID	Time	ET (hrs)	Pb (mmHg)	Self-Report					Clinical Assessment			Functional
				Head-ache	GI Symp	Fat/ Weak	Diz/ Light	Sleep Dis	Mental	Ataxia	Edema	Self-Report
A99546	7:00	-1.138	764	0	1	0	0	0	0	0	0	0
A99546	20:00	-0.597	764	0	0	0	1	0	0	0	1	0
A99546	7:00	-0.138	752	0	0	0	0	0	0	0	0	0
A99546	20:00	0.403	445	1	1	2	1	1	1	1	1	1
A99546	7:00	0.862	445	2	2	2	2	3	2	3	2	3

Table 3. Pre-exposure or baseline characteristics of subjects

Subject ID (random assignment)	Gender (1=male, 2=female)	Age at start of study (y)	Height (m)	Weight (kg)	Altitude of Origin (m)	Previous Altitude Illness (0=no, 1=yes)	Nights above 2,000 m in Past Month	Climbing Experience (0=none, 1=2,000-5,000m, 2=>5,000 m
B08769	1	27	1.95	83.2	500	1	0	1

ACKNOWLEDGMENTS

Supported by U.S. Army Contract DAAD16-01-P-0559.

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Chapter 26

LATE ABSTRACTS

HIGH ALTITUDE COUGH IS NOT CAUSED BY CHANGES IN PLASMA BRADYKININ

Nicholas P Mason, Merete Petersen, Christian Mélot, Akpay Sarybaev, Almaz Aldashev, Robert Naeije

RATIONALE: Cough can occur in patients taking angiotensin converting enzyme (ACE) inhibitors due to the stimulation of airway rapidly adapting receptors by increased levels of bradykinin which is degraded by ACE. Hypoxia has been reported to decrease ACE activity. **METHODS:** 20 healthy volunteers underwent baseline (BL) tests at 700m before being transported to 3800m altitude by road (HA). ACE activity, plasma bradykinin concentration and citric acid cough threshold (CACT) were measured at BL and HA1. CACT was also measured during the 2 week stay at HA. Forced vital capacity (FVC); transepithelial nasal potential difference (NPD) and electrical impedance tomography (EIT) measurements reflecting respiratory epithelial ion transport and extravascular lung water, respectively, have been published from these same subjects (Mason NP *et al*, J. Appl. Physiol. Published online Dec 2002). **RESULTS:** There was no change in ACE activity on ascent to altitude (41.87±4.0 vs 41.79±3.9 mU ml^{-1}, p=0.963) although plasma bradykinin levels fell at HA1 *cf* BL (0.85±0.4 vs 0.17±0.05 ng ml^{-1}, p<0.001). CACT fell on ascent to HA (5.26±1.03 vs 3.55±0.6 g l^{-1}, p=0.024). There was no correlation between ACE activity or plasma bradykinin and CACT. However Poon analysis of repeated measures during the stay at HA revealed a correlation between CACT and FVC (R^2= 0.546); EIT (R^2= 0.292) and NPD (R^2= 0.212). **CONCLUSION:** High altitude cough is not caused by changes in plasma bradykinin levels, but Poon correlations between CACT and FVC, EIT and NPD would be consistent with an aetiological role for subclinical pulmonary oedema due to altered respiratory epithelial ion transport.

MODERATE HIGH ALTITUDE CAUSES SUSTAINED AND MARKED SYMPATHOEXCITATION

Mikael Sander, Carsten Lundby, José Calbet, Gerrit van Hall

During continued exposure to hypobaric hypoxia increasing norepinephrine levels indirectly indicate sympathoexcitation. **Aim:** 1) to provide direct microneurographic evidence for sympathoexcitation during acclimatization to moderate high altitude; and 2) to test the

Hypoxia: Through the Lifecycle, edited by R.C. Roach *et al.*
Kluwer Academic/Plenum Publishers, New York, 2003.

hypothesis that decreased nitric oxide (NO) production is mechanistically involved. **Methods and results:** In 8 Danish lowlanders we measured mean arterial pressure (MAP), heart rate (HR) and muscle sympathetic nerve activity (MSNA) twice at sea level (normoxia and acute hypoxia (12.6 % oxygen)) and twice in high altitude (after 10 and 50 days at 4.100 m in El Alto, Bolivian Andes). Measurements were also obtained in 8 Bolivian highlanders (born and living at 4.100 m). As expected, this level of acute hypoxia used caused no chemoreflex activation (MSNA: 15±2 vs. 16±2 bursts/min). The major new finding is markedly elevated MSNA levels during acclimatization to moderate high altitude. This was accompanied by increases in MAP and HR. Data for sea level vs. 10 and 50 days in high altitude: MAP: 72±2 vs. 78±2 and 75±2 mmHg; HR: 54±3 vs. 67±3 and 65±3 beats/min; MSNA: 15±2 vs. 42±5 and 42±5 bursts/min, all $p<0.05$. The Bolivians had high levels of MSNA (34±4 bursts/min), but MAP and HR were similar to Danes at sea level. The NO synthase substrate, L-arginine (200 mg/kg intravenously), caused no significant changes in MAP, HR or MSNA at sea level or high altitude. **Conclusions:** Simultaneous increases in MAP, HR and MSNA indicate sustained sympathetic overactivity during acclimatization to moderate levels of hypobaric hypoxia, which does not acutely engage chemoreflexes. We could find no evidence that decreased NO production plays a mechanistic role. Further study of this novel model of sympathoexcitation may improve our understanding of the mechanisms underlying sympathoexcitation in conditions such as heart failure and obstructive pulmonary disease.

PROPHYLAXIS OF HIGH ALTITUDE ILLNESS TRIAL (PHAIT): GINKGO MAY WORSEN SYMPTOMS OF ACUTE MOUNTAIN SICKNESS (AMS) IN HIMALAYAN TREKKERS

JH Gertsch, Basnyat B, Johnson W, and Onopa J for the authors of the Prophylaxis of High Altitide Illness Trial (PHAIT)

Small trials have reported that Ginkgo biloba is an effective prevention for symptoms of acute mountain sickness, but to date there have been no large randomized trials comparing Ginkgo with acetazolamide (ACET) for AMS. In a prospective, double-blind, randomized, placebo-controlled trial we compared Ginkgo 120 mg BID, ACET 250 mg BID, ACET 250 mg plus Ginkgo 120 mg BID, and placebo for prevention of AMS. Trekkers were enrolled at 4248m or 4397m, and took 3-4 doses before ascending to 4950m, where data was collected. 591 trekkers were enrolled, 488 completed the trial, and 103 were lost to follow-up (17.4%). The incidence of AMS in the entire cohort was 208 of 488 (42.7%). AMS incidence were: placebo, 64 of 119 (53.8%); Ginkgo, 76 of 124 (61.3%); ACET, 25 of 118 (21.2%); and Ginkgo+ ACET, 43 of 126 (34.1%). AMS severity was measured as Lake Louise raw scores that were low (4 or less) or high (5 or greater). In the placebo group, 43 (25.1%) had high scores and 76 (63.9%) were lower. In the Ginkgo group, 47 (37.9%) had high scores, and 77 (62.1%) were lower. In the ACET group, 13 (11.0%) had high scores, and 105 (89.0%) were lower. In the Ginkgo+ ACET group, 19 (15.1%) had high scores, and 107 (84.9%) were lower. The headache incidence in the entire cohort was 272 of 488 (55.9%). Headache incidence in the groups was: placebo, 81 of 119 (68.1%); Ginkgo, 92 of 124 (74.2%); ACET, 36 of 118 (30.5%); Ginkgo+ ACET, 63 of 26 (50.0%). These results

were statistically significant (p= <0.05) and suggest that, contrary to the findings in our previous study, Ginkgo was not effective at preventing AMS, and may worsen illness.

INCREASE IN SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR AFTER INTERMITTENT HYPOXIC TRAINING IN NATIVE HIGH-ALTITUDE ATHLETES

Ri-Li Ge, Hai-Ping liu, Fu-Hai Ma, Rong-Yun Fan, Yan-Hong Hui, Gou-En Jing, Ying-Zhung Yang.

Intermittent hypoxic training has been proposed to enhance exercise performance in sea level athletes, but is unknown in native high-altitude athletes. The present study was performed to investigate the effects of the intermittent hypoxic training on the vascular endothelial growth factor (VEGF),which is a potent angiogenic cytokine, and erythropoietin (EPO) in native high-altitude athletes. Ten prefrontal endurance runners (6 male 4 female) were sleeping at simulated altitude of 4000m (8 hours each night; from 10pm to 6am) for four weeks. At the daytime, they were training intensely at 2,260m. Another 10 athletes were sleeping and training at 2,260 m as a control. All athletes of both groups were born and living at moderate altitudes (from 2300m to 3000m), and well matched with age, sex, training intensity. The serum VEGF, EPO, and transferring receptor (TFR) were measured before, during 4weeks of night altitude exposure, and 2 weeks after hypoxic exposure. VEGF concentration at the base line in hypoxia and control groups was 32.54 ± 7.07 pg/ml and 43.87 ± 13.24 pg/ml, respectively. It was slightly increased by 3 and 4 weeks of hypoxia exposure (10.25% and 27.5%), and significantly increased (48.86%) at the end of one week after finishing hypoxic exposure (i.e., at the 5 weeks). The increase in VEGF varied markedly among individuals, ranging from -25% to 110% at the fifth week. In the control group, there was no significant change in the VEGF either intermittent hypoxic training or normoxic training. Both EPO and TFR showed no changes during and after hypoxic training in both groups. These data suggest that 1) intermittent altitude training can induce VEGF release, which may increase the oxygen delivery in skeletal muscle tissue; 2) VEGF release to altitude exposure may be more sensitive than the EPO release in native high-altitude athletes, suggesting that there are different physiological responses between sea level athletes and high altitude athletes. This work was funded by Natural Science foundation of China No.30140011.

HIF-1α EXPRESSION IN SKELETAL MUSCLE CONTROLS SYSTEMIC EXHAUSTION

Steven D. Mason, Matthew J. Kim, Richard A. Howlett, Wayne D. McNulty, I. Mark Olfert, Reed Hickey, C. Ronald Kahn, Frank J. Giordano, Michael C. Hogan, Peter D. Wagner, and Randall S.Johnson

Muscle tissue experiences tremendous changes in metabolic flux from rest to intense exertion. This in turn affects the relationship between oxygen availability and utilization, with classically studied effects on the glycolytic pathway and lactate accumulation. Lowered

oxygen levels, or hypoxia, induce the expression of the transcription factor HIF1χ, which regulates a coordinated transcriptional response; that response includes up regulation of the glycolytic pathway and the angiogenic factor VEGF. We have targeted deletion of HIF-1α to skeletal muscle to determine how sensation of oxygen regulates muscle function during exercise. In addition to deficiencies in metabolic substrate utilization and gene expression, we found that the knockout of HIF-1α caused a dramatic increase in whole animal exercise endurance, as measured by both treadmill and swimming tests. This increase was correlated with decreased lactate accumulation in exercising animals both intra-muscularly and at the level of serum accumulation. Intriguingly, we found that this increased capacity for exercise in HIF-1α knockout mice also causes increased muscle damage after exercise; this is dramatic evidence that sensation of fatigue through lactate accumulation is an essential mechanism for avoidance of tissue damage. Our data demonstrate that HIF-1α is a key regulator of whole animal endurance, and that sensation of oxygen levels is critical for preventing muscle damage during extended activity.

ALVEOLAR VENTILATION: AN EXAMINATION FROM FIRST PRINCIPLES

Ron Somogyi, Hiroshi Sasano, Takafumi Azami, Alex Vesely, David Preiss, Eitan Prisman, Steve Iscoe, Joseph Fisher.

Introduction: Sommer *et al* (*Eur Respir J* 12:698-701, 1998) described a breathing circuit to maintain isocapnia independent of increases in minute ventilation (VE). The circuit delivers a flow of fresh gas (FGF, equal to resting VE) followed by, if actual VE exceeds resting VE, reserve gas containing some CO_2. The PCO_2 of the reserve gas ($P_{RG}CO_2$) is such that it does not participate in CO_2 exchange in the alveoli. In practice, however, when VE increases above resting levels, there is an initial drop in end-tidal PCO_2 ($P_{ET}CO_2$) followed by an increase above control. Our equation VA = FGF + (VE – VDAN - FGF) x ($P_{ET}CO_2$ - $P_{RG}CO_2$ / $P_{ET}CO_2$) (where VA is alveolar ventilation and VDAN is ventilation of the anatomic dead space) predicts optimal control of $P_{ET}CO_2$ when FGF = V_E -VDAN (where VDAN = 2 ml/kg/min) and $P_{RG}CO_2$ is equal to control $P_{ET}CO_2$. **Purpose**: To compare the settings recommended by our model to those of Sommer *et al.* (FGF = VE; $P_{RG}CO_2$ = mixed venous PCO_2). **Method:** Five male subjects breathed via the circuit for 5 min each at resting VE and at 2-5 × resting VE. FGF and $P_{RG}CO_2$ were set according to our model and as recommended by Sommer *et al.* **Results:** Using the settings recommended by Sommer *et al.*, the maximal reduction in $P_{ET}CO_2$ from control (40.1 ± 0.6 mmHg) was 1.8 mmHg ($p = 0.01$) at 1.75 × resting VE and rose by 4.1 mmHg ($p < 0.001$) at 5 × resting VE. With optimal settings according to our model, $P_{ET}CO_2$ did not change from control ($p = 0.1$) at any VE. **Conclusion:** During increases in VE, $P_{ET}CO_2$ varied less using settings of FGF and $P_{RG}CO_2$ based on our model.

INTERMITTENT HYPOXIC TRAINING (IHT): EFFECTS ON HEMATOLOGICAL AND PERFORMANCE MARKERS IN ELITE DISTANCE RUNNERS

Colleen G. Julian, Benjamin D. Levine, James Stray-Gundersen, Christopher J. Gore, Randall L. Wilber, Jack T. Daniels, Michael Fredericson

PURPOSE: To evaluate the efficacy of short-burst intermittent normobaric hypoxia at rest as a stimulus to physiological, hematological, and performance measures. **METHODS**: National class distance runners (n=14 men) completed a four-week regimen (5:5 min hypoxic: normoxic ratio for 70 min, 5 times per wk) of either intermittent normobaric hypoxia (HYPOX) or placebo control (NORM) at rest. The study was controlled using a matched pairs, randomized, double-blind design. Subjects were matched by VO_2max, time trial performance and training history. The experimental group (n=7) was exposed to a graded decline in FIO2 (wk 1: = 0.12, wk 2: = 0.11, wk 3 and 4: = 0.10). The placebo control group (n=7) was exposed to the same temporal regimen, but breathed a FIO_2= 0.209 for the entire 4 wk period. Primary measures were 3,000m track time trial, VO_2max, hemoglobin, hematocrit and reticulocyte count, all repeated twice at baseline, and immediately and 3 wk after exposure. Oxygen saturation measurements were recorded by a blinded observer for each subject at the conclusion of the initial session of each hypoxia/normoxia stage: mean ± SD week 1: 90% ± 4/ 97% ±2; week 2: 86%±5/97%±1; week 3: 86%±4/ 97%±1; week 4: FIO_2% 81%±4/97±1. **RESULTS**: see table for mean ± SD; no significant differences were found for any comparison. **CONCLUSION**: Four weeks of IHT does not improve performance nor change hematological measurements in elite distance runners.

THE EFFECT OF DIFFERENT INSPIRED OXYGEN FRACTIONS ON ARM EXERCISE PERFORMANCE

Maria T.E. Hopman, Hans T.M. Folgering, Jan T. Groothuis, Sibrand Houtman

It has been shown that peak oxygen uptake (VO_2peak) during leg exercise is enhanced with an increased inspiratory oxygen fraction indicating that oxygen supply is the limiting factor during dynamic exercise with a large muscle mass. Whether oxygen supply is a limiting factor in arm exercise, i.e., dynamic exercise with a small muscle mass, is unknown. The purpose f this study, therefore, was to examine the effect of different levels of FiO_2 on VO_2peak during arm exercise in healthy individuals. Nine men successfully performed three incremental arm-cranking exercise tests until exhaustion, with $FiO_2$15%, $FiO_2$21% and $FiO_2$50% applied in counterbalanced order on three different days, separated by one week. A significant FiO_2 dependency (meaning that there is a direct relationship for this variable with the three levels of oxygenation) was observed for VO_2peak (p=0.02) and power output (p=0.01) and post-hoc tests revealed a significant difference in VO_2peak between 15 and 50% FiO_2 (p=0.02), but not between 15 and 21% FiO_2, and 21 and 50% FiO_2. The results of this study show that arm exercise performance is enhanced with increasing FiO_2, and suggest that VO_2peak during arm exercise is limited by oxygen supply, rather than by the metabolic machinery within the muscle itself.

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