ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 588

HYPOXIA AND EXERCISE

Edited by
Robert Roach
Peter Wagner
and
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HYPOXIA AND EXERCISE 2007
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HYPOXIA AND EXERCISE

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The 14th International Hypoxia Symposium was dedicated to the memory of Dr. John T. "Jack" Reeves.

As most of you know, Dr. John T. "Jack" Reeves passed away in September, 2004 following a motor vehicle-bicycle accident. Jack was a former Professor of Medicine, Pediatrics and Surgery and Professor Emeritus at the University of Colorado Health Sciences Center, having joined the faculty in 1972. He made exceptional contributions in teaching/mentoring, research, administration and leadership. He was a scientist of international stature. He made major advances at the molecular, cellular, animal and human level with regard to the pulmonary circulation and adaptation to high altitude. For many years Jack was a senior member of the Cardiovascular Pulmonary Laboratory of the School of Medicine within the Department of Medicine and most recently played a significant role in the establishment of the Colorado Center for Altitude Medicine and Physiology (CCAMP). He was a key, active and memorably vocal member of the Hypoxia family. No one who ever met Jack at Hypoxia will forget him; his spirit lives on within the Hypoxia community.

In honor of Jack, we established The Reeves Prize for Presentation Excellence. This prize will be awarded at the biennial International Hypoxia Symposium to the speaker judged to present the most outstanding scientific talk, with special emphasis on clarity of presentation skills that were cherished, taught and practiced by Jack. The recipient will be named at the closing banquet by the organizers of the International Hypoxia Symposia with funds coming from the John Sutton Fund, McMaster University, the Colorado Center for Altitude Medicine and Physiology, University of Colorado at Denver and Health Sciences Center, and the International Hypoxia Symposia. The winner for 2005 was Dr. Randy Sprague. See his written contribution starting on page 207.

PREFACE AND ACKNOWLEDGEMENTS

The International Hypoxia Symposia convenes every other year to bring together international experts from many fields to explore the state of the art in normal and pathophysiological responses to hypoxia. Representatives from 18 countries joined together in February 2005 for four days of intense scientific discourse in the dramatic mountain setting of Lake Louise, Canada.

The 14th International Hypoxia Symposium was a rewarding experience due to the outstanding faculty and the lively participation of our largest-ever group of participants. At this, our fourth 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.

As editors of the Proceedings of the International Hypoxia Symposia, we strive to maintain a 28 year tradition of presenting a stimulating blend of clinical and basic science papers focused on hypoxia. Topics covered in 2005 included a history of high altitude physiology, arterial hypoxemia in exercise, sleep and hypoxia, genetic components of adaptation to hypoxia, erythropoietin, cell stress pathways and hypoxia, the role of red blood cells, ATP release and hemoglobin in the control of vasoreactivity, exercise and altitude training, the eye at high altitude and pulmonary hypertension in high altitude residents. An update on methods to measure the hypoxic ventilatory response was also featured.

The abstracts from the 2005 meeting were published in High Altitude Medicine & Biology Dec 2004, Vol. 5, No. 4: 471-509. Late abstracts are presented staring on page 311, this volume.

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.

In 2005 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, our International Advisory Committee. At the meeting we were greatly helped by Barbara Lommen, Paige Sheen, Mollie Pritcher, Gene and Rosann McCullough and Andy Subudhi who each made a tremendous effort to make every delegate feel at home, and to make the meeting go very smoothly.

Please join us by the light of the full moon in February 2007 at the Chateau Lake Louise, Lake Louise, Alberta, Canada for the 15th International Hypoxia Symposium.

Robert C. Roach, Peter D. Wagner, Peter H. Hackett, Editors, Fall 2005
(www.hypoxia.net)

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Chapter 1

A TRIBUTE TO JOHN BURNARD WEST

James S. Milledge
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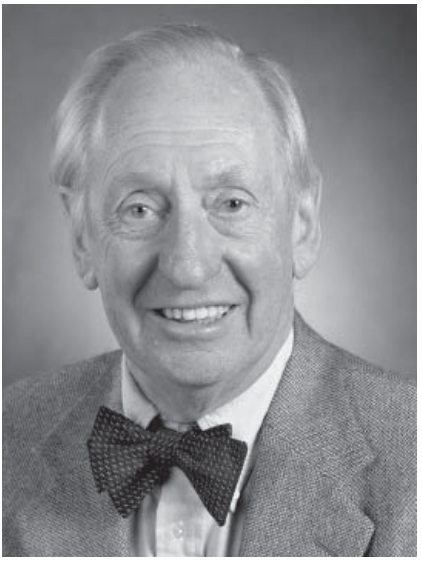

Abstract: John West is well known to the "Hypoxia" community for his many contributions to the physiology and Pathophysiology of high altitude and for his leadership of the 1981 American Medical Research Expedition to Everest. He is known to the wider medical world for his researches into respiratory physiology especially gas exchange in the lung and perhaps even more for his numerous books on these topics. His publication list numbers over 400 original papers. His research career started in the UK but since 1969 he has been Professor of Medicine at UCSD, leading a very productive team at La Jolla. He has been honoured by numerous prizes and named lectureships, the latest honour being to be elected to the Institute of Medicine, National Academies (USA).

Hypoxia and Exercise, edited by R.C. Roach *et al.*
Springer, New York, 2006.

Key Words: biography, gas exchange, altitude physiology, altitude pathology, cardio-respiratory physiology, space medicine

INTRODUCTION

It is entirely fitting that this year when he has recently been honoured by being elected a Member of the Institute of Medicine, National Academies, the Symposium organisers should select John West to be the scientist who we are honouring. I am pleased to be asked to compose this tribute but if it is not as fulsome and laudatory in tone as some previous ones, I ask John's indulgence and plead the twin handicaps of the British love of understatement and an old friend's licence to tell it as he sees it!

I have known John West as a friend, expedition companion and scientific colleague for about 45 years. We first met in 1960 when preparing for the 1960-61 Himalayan Scientific and Mountaineering Expedition, popularly known as the Silver Hut Expedition. I was, even then, aware of his reputation as a bright young medical researcher at the Postgraduate Medical School, Hammersmith Hospital where he had done work on lung gas transfer, exploiting the unique opportunity afforded by the MRC cyclotron sited there.

Since then our paths have crossed and re-crossed. He invited me on his American Medical Research Expedition to Everest (AMREE) in 1981, and we were together on a trek in Sikkim in 2000. From 1985 to the present we have worked as joint authors, with Michael Ward, on the textbook "High Altitude Medicine and Physiology" as well as frequent meetings at conferences and social occasions.

John is a man of many parts, researcher, teacher, author, editor, and organiser/administrator. His interests also include music, ham radio, radio-controlled gliders, tennis, skiing, mountaineering and of course he is a family man.

In this short tribute I can only give the reader a flavour of the man and a few personal reminiscences. No doubt there will, in time, be a full biography of John B. West!

The Researcher

John West is probably best known to the Hypoxia community for his research work in the field of high altitude medicine and physiology. His interest in the effects of altitude hypoxia followed naturally from his early work on pulmonary gas exchange which started when he was a junior doctor at the Postgraduate Medical School. The MRC cyclotron could produce short-lived radio-isotopes of oxygen. The oxygen-15 could be inhaled or infused either as O_2 or CO_2 and the activity counted over the chest wall. This resulted in a number of important papers in 1960. The one I remember reading at the time I first met John was in the *BMJ*. There was also one in *J. Appl. Physiol.* in the same year. This was the time when I was working with Dr Griffith Pugh (honoured at Hypoxia 1993) preparing for the Silver Hut Expedition of which he was the Scientific Leader. John had also been invited to be a member and we met doing base line exercise experiments at Griff's MRC lab in Hampstead and again in Oxford for control of breathing studies, my particular responsibility (Figure 2).

The work John did on the Silver Hut was again on gas exchange. He measured the diffusing capacity of the lung for carbon monoxide and showed there to be no change with acclimatization, apart from the small effect of increased haemoglobin concentration. He

was also involved, as we all were, in the exercise studies and with Mike Ward measured $\dot{V}O_2$ max on the Makalu Col (7440m), still, 44 years later, the highest altitude at which it has been measured! (Figure 3) The reduction in $\dot{V}O_2$ max with altitude, he showed, was largely due to diffusion limitation, again a matter of gas exchange.

Figure 2. John West as subject of a control of breathing experiment inside the Silver Hut, 1961.

Figure 3. John West and Michael Ward setting up the bicycle ergometer on the Makalu Col, (7440m) to measure $\dot{V}O_2$max on themselves.

After the Silver Hut, he did a post-doc fellowship with Herman Rhan (an Honouree at Hypoxia 1991) in Buffalo and then became leader of a MRC Respiratory Physiology Group at the Hammersmith studying pulmonary gas exchange. He had a sabbatical year 1967-8 with NASA at the Ames Research Center. Here he has the opportunity to work on computer models of gas exchange. He put in his first research proposal to NASA on lung function in astronauts at the end of this year and since then has had a continuing connection with space research on the effect of micro-gravity on gas exchange.

In 1969 John joined the faculty of University of California, San Diego where he is still working as Professor of Medicine. On arrival, his NASA project was approved and a check for $100,000 started his career in La Jolla on the right foot! Work on computer modelling of gas exchange led, in collaboration with Peter Wagner, to the multiple inert gas method for measuring V/Q ratio inequalities in health and disease. His more recent research includes work on stress failure of the alveolar-capillary wall as part of the mechanism of high altitude pulmonary edema and the use of oxygen enrichment of room air in high altitude living quarters.

Publications

John's bibliography is impressive and can be found on the web at: http://medicine.ucsd.edu/faculty/west/.

He has published 424 papers, in 290 of which he is the first author (68.4%). The great majority of these have been in prestigious peer reviewed journals. By today's standards he was a late starter. His first paper, on ventilation-perfusion ratio inequality in the lung by single breath analysis, was in *Clinical Science* in 1957 when he was 28 and had been qualified for 6 years. However once John got started, he was soon churning out significant papers in respectable numbers as shown in Fig 4.

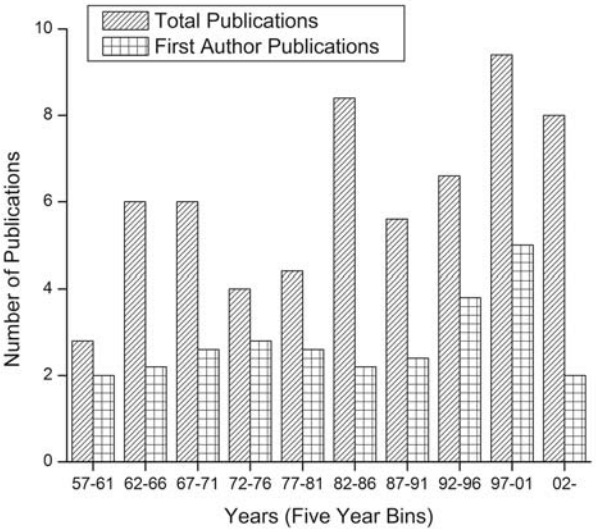

Figure 4. Papers published by John West, average number per year in 5 year bins, showing 1st author and totals.

As impressive as the numbers and quality of his papers, is the fact that with the passing years his output, far from diminishing, has actually increased! There is a significant linear increase in numbers of paper with each quinquennium of his career ($p=0.015$). If the trend continues, I can confidently predict, that on reaching his centenary, he will be publishing at the rate of 16.5 papers per year!

The Teacher

I cannot report on John's ability as a teacher in medical school though I am sure his courses are very well appreciated. As a lecturer I have heard him on numerous occasions and can vouch for his ability to give a clear and exciting account of even the most difficult of subjects such as *V/Q ratio inequalities*. His lecture on AMREE and Peter Hackett's solo summit climb is exciting in both scientific and mountaineering content and style. Evidence of the widespread appreciation of his abilities as a lecturer is the number of prestigious named lecturers he has been asked to give: 57 at the last count, mostly in the USA but also in UK, Canada, Australia and Russia.

However, I think that his most important role as a teacher is as author of numerous textbooks and monographs. These have made him well known to the wider medical world. John has 21 books on his publications list; in some, he is the editor of multi-author books or conference proceedings but in the majority he is sole author. The titles range from *Ventilation/Blood flow and Gas Exchange,* his first monograph, through *The Use of Radioactive Materials in the Study of Lung Function; Translations in Respiratory Physiology*, his first assay into the history of our subject, to *Gravity and the Lung: Lessons from Microgravity,* his latest book. For the Hypoxia community, John's excellent history of our subject, *High Life,* and our joint book *High Altitude Medicine and Physiology* are, no doubt the best known. However, John's most influential book is probably *Respiratory Physiology - The Essentials* followed by *Pulmonary Pathophysiology - The Essentials*. The former was originally written for Medical Students and both are required reading for many postgraduate courses. Both books have been translated into many languages and have gone into 7 and 6 editions in English, respectively. Mention John West's name to any young anaesthetic trainee and (s)he is likely to respond, "Not *The* John West of *Respiratory Physiology*!"

Also important in the wider teaching role of promoting scientific communication, is John's work in the thankless task of editorship. He showed early promise of this by starting a journal whilst still a school boy at Prince Alfred College in Adelaide. The PAC Science Journal is still being published 60 years later! John is on the editorial board of 17 learned journals and of course is the Editor-in-chief and founder of our own, very successful journal, *High Altitude Medicine and Biology.* We all hope his latest journal proves to be as long lived as his first!

The Man

He chose to head up quite a small division of physiology at La Jolla rather than build up a big empire but his organisational abilities are considerable. Anyone who can put together an Everest Expedition, especially one that carries through both scientific and mountaineering objectives as he did with AMREE has to have such skills in spades! He has received numerous honours including being elected President of the American Physiological Society and the International Society of Mountain Medicine. His most recent honour, last year, is to have been elected to the Institute of Medicine of the National Academy of Sciences (USA)!

I personally owe John a lot, in that he was one of three or four men who influenced me in becoming interested in academic medicine and specifically respiratory physiology. We had many long discussions whilst trekking on the Silver Hut Expedition or sharing a tent in the Western Cwm on AMREE. One of the earliest of these I remember was his unfolding to me the beauties of the Rhan/Otis O_2/CO_2 diagram! Happy days!

John never claimed to be a climber but he has a love of mountains and enjoys trekking and skiing. The pinnacle of his mountaineering was probably, at the end of the Silver Hut Expedition. He made a solo ascent to the Makalu Col and organised the rescue of the near dead climber, Pete Mulgrew, from the Col when other members of the team were exhausted and devoid of drive.

John has been fortunate in having a wonderfully supportive wife in Penny and two children to be proud of in Joanna and Robert.

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Chapter 2

ADVENTURES IN HIGH-ALTITUDE PHYSIOLOGY

John B. West
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Abstract: I have probably had more fun doing high-altitude physiology than most people. Some 45 years ago I applied to be a member of Sir Edmund Hillary's Silver Hut expedition and was accepted in spite of having no previous climbing experience. On this project a group of physiologists wintered at an altitude of 5800 m just south of Everest and carried out an extensive research program. Subsequently measurements were extended up to an altitude of 7440 m on Makalu. In fact the altitude of these field measurements of $\dot{V}O_{2max}$ has never been exceeded. This led to a long interest in high-altitude medicine and physiology which culminated in the 1981 American Medical Research Expedition to Everest during which 5 people reached the summit and the first physiological measurements on the summit were made. Among the extraordinary findings were an extremely low alveolar PCO_2 of 7-8 mmHg, an arterial pH (from the measured PCO_2 and blood base excess) of over 7.7, and a $\dot{V}O_{2max}$ of just over one liter/min. More recently a major interest has been the pathogenesis of high altitude pulmonary edema which we believe is caused by damage to pulmonary capillaries when the pressure inside some of them increases as a result of uneven hypoxic pulmonary vasoconstriction ("stress failure"). Another interest is improving the conditions of people who need to work at high altitude by oxygen enrichment of room air. This enhances well-being and productivity, and is now being used or planned for several high-altitude telescopes up to altitudes of 5600 m. Other recent high-altitude projects include establishing an international archive on high-altitude medicine and physiology at UCSD, several books in the area including the historical study *High Life*, and editing the journal *High Altitude Medicine & Biology*.

Key Words: Everest, stress failure, oxygen enrichment, gas exchange, history

INTRODUCTION

Naturally it is a great pleasure and honor to be recognized in this way by the 14th Hypoxia Symposium. I have attended essentially all of the symposia since the first at Banff

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Springs Hotel in 1979 and on every occasion there has been a very special mixture of science and camaraderie. Long may the tradition continue.

I want to take the opportunity to relate some of the high points in a career in high-altitude physiology. This talk is directed at the young people in the audience who are starting their careers. The gray hairs in the front row know most of this but I hope will indulge me.

SILVER HUT EXPEDITION

There can be few introductions to a new area of science as dramatic as being invited to join Sir Edmund Hillary's 1960-1961 Himalayan Scientific and Mountaineering Expedition now universally known as "Silver Hut". In 1959 I had had several years of training in respiratory physiology, first at the Medical Research Council Pneumoconiosis Research Unit in South Wales, U.K. and then at the Postgraduate Medical School, Hammersmith Hospital, London. But I knew almost nothing about high-altitude physiology and, apart from some skiing, had never been on a mountain. It happened that I was sitting next to someone at a meeting of the (British) Physiological Society when she happened to remark that Griff Pugh was arranging a medical research expedition to the Himalayas. I knew something of Griff because he had been the physiologist on the first successful ascent of Everest some six years before. On a whim I decided to approach him and to my astonishment he invited me to join the expedition of which he was scientific leader. Of course Ed Hillary also wanted to interview everybody before taking them on. The story I tell is that I met him in London and he asked me to climb a flight of stairs whereupon he said "Please join us." This may be apocryphal. However the whole process was a remarkable piece of serendipity for a 30 year old.

The expedition was in three parts (1, 4). In September of 1960 a large group walked in to the Everest region carrying pieces of the Silver Hut which was then erected on a glacier at an altitude of 5800 m (19,000 ft) about 10 miles south of Everest (Figure 1). A group of about 7 physiologists then lived in the Silver Hut during the winter studying the effects of acclimatization at this very considerable altitude over several months. Happily two of these are here today including Jim Milledge and Sukamay Lahiri (Figure 2). In the spring the third phase of the expedition took place when we were rejoined by the climbers and the group moved across to Makalu, the fifth-highest mountain in the world at 8481 m. The plan was to try and climb this without supplementary oxygen but a severe illness in one of the climbers near the summit prevented this.

Interestingly some new information on the planning of the expedition has recently come to light. Pugh's daughter, Harriet Tuckey, is writing a biography of her father and she has been mining the Pugh archive at UCSD (see below). She came across some correspondence in 1958 between Pugh and Philip Hugh-Jones about a possible physiological expedition to Kamet (7756 m) in the Garhwal Himalayas about 300 km northeast of New Delhi. Kamet was one of the peaks that attracted the remarkable early British mountaineer/physiologist Alexander Kellas (1868-1921) and he carried out physiological studies on Kamet in 1920 (5, 7). However funding for a major physiological expedition would have been very difficult to come by at that time, and in the event Pugh found himself with Hillary on an expedition in Antarctica in 1959 where apparently the plan of the hybrid scientific and mountaineering expedition was hatched. Remarkably Hillary was successful in obtaining

almost all of the funding for the expedition from the American publishers of *World Book Encyclopedia* (1).

There is no space here to do justice to the science of the Silver Hut expedition which has been the subject of at least two communications in previous Hypoxia Symposia (3, 8). In the Silver Hut itself there were extensive studies of exercise, pulmonary gas exchange including arterial oxygen saturation and the diffusing capacity of the lung, control of ventilation, blood studies, electrocardiogram, basal metabolism, weight loss, intestinal and psychomotor function. When the party moved to Makalu the physiological studies were extended up to Camp 5 on the Makalu Col at 7440 m where maximal exercise and electrocardiograms were studied, and alveolar gas samples were obtained as high as 7830 m. The Silver Hut expedition was the most ambitious and successful high-altitude field expedition of its time and a number of its conclusions are still cited.

Figure 1. Silver Hut at an altitude of 5800 m.

Figure 2. Four of the physiologists on the Silver Hut expedition. Left-to-right: Sukhamay Lahiri, West, Griffith Pugh, Jim Milledge. The photograph was taken in 1977.

1981 AMERICAN MEDICAL RESEARCH EXPEDITION TO EVEREST

Of all the projects in my academic career, none has given more satisfaction than the privilege of leading this expedition which obtained the first physiological measurements at the highest point in the world. Following the success of the Silver Hut expedition I often wondered whether it might be possible to make measurements high on Mt. Everest. The data point for maximal oxygen consumption that Mike Ward and I obtained on the Makalu Col (7440 m) (Figure 3) renewed the intriguing question originally posed by Kellas in 1921: can a human being reach the Everest summit without supplementary oxygen? The answer came in 1978 in dramatic fashion when Messner and Habeler made their memorable ascent, a feat that has now been repeated many times.

In many respects AMREE owed a lot to Silver Hut. For example the design of the Base Camp laboratory at 5400 m was a smaller version of the Silver Hut structure. However whereas the principal scientific question of the Silver Hut expedition was what physiological changes take place in lowlanders when they are exposed to an altitude of 5800 m for several months, the question we hoped to answer on AMREE was what physiological adaptations allow humans to reach the summit of Mt. Everest.

Again it is impossible to do justice to the science here. However the general plan of the expedition was to place laboratories at the Base Camp (5400 m), Camp 2 (6300 m) and if possible carry out some measurements at the highest Camp 5 (8050 m). Each of these sites had its own experimental projects. However in a sense they were all focused on the central aim of getting measurements at the highest possible altitude, hopefully the summit. In this the expedition was extremely lucky and a number of measurements were indeed made at an altitude of 8050 m and above, including the summit (Figure 4). Some of the most dramatic results are shown in Table 1 where it can be seen that on the summit the extreme hyperventilation reduced the alveolar PCO_2 to 7-8 mmHg with an extraordinary respiratory alkalosis and pH (based on the alveolar PCO_2 and measured base excess) of over 7.7 (11). The maximal oxygen consumption measured on extremely well-acclimatized climbers with an inspired PO_2 of 43 mmHg corresponding to the Everest summit was just over 1 liter.min^{-1} (10). It is a lucky person who can be involved an experiment like this once in a lifetime.

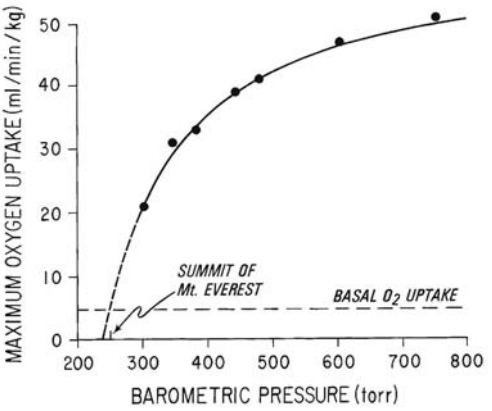

Figure 3. Maximal oxygen consumption plotted against barometric pressure at different altitudes. The lowest point was obtained on the Makalu Col (7440 m). Extrapolation of the line suggests that all the oxygen available on the Everest summit will be required for basal metabolism. These were the data available prior to AMREE.

Figure 4. Chris Pizzo, M.D. sitting on the summit of Mt. Everest collecting samples of alveolar gas.

Table 1. Alveolar gas and estimated arterial blood values on the summit of Mt. Everest.

	BAROMETRIC	INSPIRED	ALVEOLAR	---ARTERIAL VALUES---		
ALTITUDE	PRESSURE	PO2	PO2	PO2	PO2	pH
m	mmHg	mmHg	mmHg	mmHg	mmHg	
8848 (summit)	253	43	35	28	7.5	>7.7
Sea level	760	149	100	95	40	7.40

PATHOGENESIS OF HIGH-ALTITUDE PULMONARY EDEMA

Of course the opportunities for field experiments such as Silver Hut and AMREE are rare and most of us spend most of our time in the more humdrum environment of the laboratory. But in some way this next project has given me as much satisfaction as any, particularly as it started with a seemingly simple question but has progressed to an intriguing biological problem.

In the late 1980s the pathogenesis of high-altitude pulmonary edema (HAPE) was a puzzle. There was a wealth of evidence that pulmonary hypertension played a vital role. For example catheterization studies showed high pulmonary artery pressures in patients with HAPE, susceptible individuals tended to have an unusually strong hypoxic pulmonary vasoconstriction response, pulmonary vasodilator drugs were useful both for treatment and prevention, and a restricted pulmonary vascular bed was a risk factor as was exercise. Then in 1986 Brownie Schoene and Peter Hackett boldly performed bronchoalveolar lavage on climbers with HAPE high on Denali and made the critical observation that the edema fluid was of the high-permeability type (6). This immediately suggested that the capillary wall was damaged and raised the question of whether the pulmonary hypertension could be the mechanism.

Nobody had previously exposed pulmonary capillaries to graded increases in hydrostatic pressure and examined them by electron microscopy for ultrastructural changes. When Odile Mathieu-Costello and I did this we were astonished to see obvious disruptions of the capillary endothelium and alveolar epithelium at pressures that we believed could occur in HAPE (Figure 5). Incidentally, it is perhaps surprising that no one has bothered to repeat these ultrastructural studies that were first published 14 years ago. We then recognized a

feature of pulmonary capillaries that had apparently previously been overlooked, namely because of the excruciatingly thin blood-gas barrier required for gas exchange, the wall stresses become enormous at high capillary pressures. We therefore coined the term "stress failure" which was borrowed from engineering, and we stated in 1991 that this could explain the pathogenesis of HAPE (12). This explanation has stood the test of time.

But the project did not stop there. One day while we were doing experiments someone walked into the lab and asked us whether we knew that racehorses bled into their lungs. I had never heard of this before but indeed it transpires that every Thoroughbred in training breaks its pulmonary capillaries as evidenced by hemosiderin-laden macrophages in tracheal washings (13). The reasons for this extraordinary situation is that these animals have been inbred for hundreds of years for great speed and this requires an enormous cardiac output. The horses therefore have very high left ventricular filling pressures leading to high pulmonary venous and capillary pressures. Interestingly elite human athletes also apparently develop changes in the integrity of their blood-gas barrier during maximal exercise (2). A further example of edema caused by stress failure is high states of lung inflation caused for example by PEEP in the intensive care unit.

What ultimately turned out to be of greater biological interest were questions like what is responsible for the strength of this highly vulnerable blood-gas barrier. The answer apparently is the type IV collagen in the basement membranes. Another question was how special is the makeup of the mammalian blood-gas barrier, the answer being not special at all in that the three-ply design (alveolar epithelium, extracellular matrix, capillary endothelium) has been highly conserved since animals first ventured on to land, such as the ancestors of the present-day lungfishes some 400 million years ago. Finally how is it that the blood-gas barrier is able to remain so excruciatingly thin but just strong enough to withstand the maximal physiological stresses to which it is exposed. The answer presumably is that it is being continually regulated in response to the wall stress, a lively area of research at the present time. Thus this project has been exceptionally stimulating because what appeared to be a relatively simple question on pathogenesis of HAPE has led us into much more fundamental questions of lung biology.

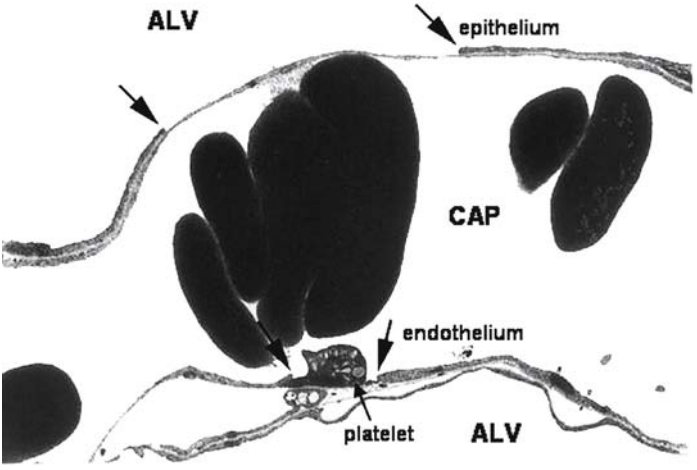

Figure 5. Ultrastructural changes in the wall of a pulmonary capillary when the capillary hydrostatic pressure was raised. The arrows at the top show a disruption in the alveolar epithelial layer; the arrows at the bottom show a break in the capillary endothelial layer with a platelet adhering to the exposed basement membrane. ALV, alveolus; CAP, capillary lumen. Modified from (12).

OXYGEN ENRICHMENT OF ROOM AIR AT HIGH ALTITUDE

In contrast to the last project, this subject has no broad biological significance. Indeed it might be argued initially that it is trivial from a scientific point of view. Yet it also has proved to be enormously satisfying because it is changing the way that people work at high altitude and, for example, makes it possible for astronomers to operate at altitudes that would be impossible without this advance.

The general principle could hardly be simpler. The detrimental effects of high altitude are caused by the low PO_2 in the air and so the obvious way to circumvent these is by raising the PO_2. Of course this can be done using portable oxygen equipment but this is cumbersome and awkward to use 24 hours a day. The solution is to add oxygen to the ventilation of the room thus raising its concentration (9). At a typical facility at an altitude of 5000 m, the oxygen concentration is raised from 21 to 27%, and this reduces the equivalent altitude (based on the inspired PO_2) to about 3200 m. Since most people working at these altitudes already have some acclimatization, an altitude of 3200 m is easily tolerated.

This procedure has some interesting technical problems. First it has only become economically feasible since the introduction of oxygen concentrators that produce oxygen from air. These are now used by the thousands in homes of people with chronic lung disease. The concentrators work by pumping air at high pressure through a tube of synthetic zeolite with the result that the nitrogen is preferentially adsorbed and the effluent gas has a high oxygen concentration. These concentrators are robust, self-contained and typically only require about 350 watts of electrical power to produce 5 $l \cdot min^{-1}$ of 90-95% oxygen. This is then fed into the ventilation duct of the room. A typical room containing 2 people at an altitude of 5000 m requires 4 concentrators. It is also possible to provide the oxygen from liquid oxygen tanks but the running costs are about ten times higher in a typical facility.

The amount of ventilation with fresh air is an important factor. Clearly the higher the ventilation, the more oxygen that has to be generated. We use the ASHRAE (American Society of Heating, Refrigeration, and Air Conditioning Engineers) 1975 standard which is 8.5 $m^3 \cdot h^{-1}$ per person. The CO_2 concentration in the room is monitored and kept at or below 0.3%. Much higher concentrations can be present without causing a health hazard or the occupants being aware of them, but the CO_2 level is a useful index of the adequacy of ventilation. Of course the oxygen concentration is also monitored.

Another important issue is the possible fire hazard. This has been carefully studied by the National Fire Protection Association and it is possible to choose a room oxygen concentration that provides substantial benefit to the occupants at high altitude but that is below the fire hazard level. It should be remembered that although the PO_2 of the air in the room at high altitude is raised by the addition of oxygen, the resulting PO_2 is always far below the sea level value.

The principal use of oxygen enrichment of room air to date has been in high-altitude telescope facilities. The longest experience has been in a radiotelescope operated by the California Institute of Technology at an altitude of 5050 m in north Chile (Figure 6). This has been in continuous operation for 5 years and the astronomers are adamant that the project could not have gone ahead without room oxygen enrichment. A number of other high-altitude telescopes are installing or planning oxygen enrichment of room air. The principle has also been used in the sleeping quarters of the Collahuasi mine which is situ-

ated at an altitude of about 4500 m though the dormitories are lower at 3800 m. Sleep is often impaired at high altitude and oxygen enrichment of room air has proved to be very valuable to some of the miners.

Rarely in my experience does a project progress from an idea to implementation in a few years. However in this case the Cal Tech astronomers were using it in their telescope less than 5 years after the initial description (9) and this certainly resulted in a warm fuzzy feeling.

Figure 6. Oxygen-enriched room at the Cal Tech radio-telescope (5050 m). The oxygen concentration in the room is 27% giving an inspired PO_2 equivalent to that of an altitude of 3200 m. Courtesy of the California Institute of Technology.

HISTORY OF HIGH-ALTITUDE PHYSIOLOGY AND A JOURNAL

The last adventure is something of a hodgepodge but no less satisfying for that. We are fortunate at UCSD to have an excellent archival library known as the Mandeville Special Collections Library. Some years ago I was talking to the librarian about depositing some material there and she suggested that we start an archive in high-altitude medicine and physiology. This was done and as far as we know is the only such archival collection in the world.

The primary purpose of an archival collection of this kind is to gather correspondence, documents, experimental protocols, laboratory notebooks, field journals and the like. Published material is of less interest because it is available elsewhere. The archive now contains a very extensive collection from Griffith Pugh which was referred to earlier. Another large collection is from Ulrich Luft, and there are various amounts of material from other workers in the field including Bruno Balke, Elsworth Buskirk, Erik Christensen, David Bruce Dill, Thomas Hornbein, Steven M. Horvath, Herbert Hultgren, Alberto Hurtado, James S. Milledge, Nello Pace, Edward J. Van Liere, Michael P. Ward, Oscar A.M. Wyss and myself. An archive like this increases in value as time passes and more material is added. The archive can be accessed on the web at <roger.ucsd.edu> and searching for the title High Altitude Medicine and Physiology Collection. Potential donors should contact

the librarian in charge, Lynda Claassen <Lynda@library.ucsd.edu>.

Another historical interest has been researching some prominent figures in high-altitude medicine and physiology including: Robert Boyle, George Finch, Stephen Hales, Alexander Kellas, and Thomas Ravenhill. This interest has been stimulated by the enlightened policy of the editors of the *Journal of Applied Physiology* who have welcomed occasional historical articles. The most ambitious product of this research has been the book *High Life: A History of High-Altitude Physiology and Medicine* which I am glad I took the trouble to write because it is so useful for reference.

A final adventure in this list has been the journal *High Altitude Medicine & Biology*. Initially I was reluctant to take this on because I thought there were enough journals and I told the publisher so. However Mary Ann Liebert was very persuasive and she convinced me that there was a niche and indeed I now think she was right. There are a number of articles in the area that are important but do not fit easily into existing clinical, physiological or other biological journals. It was gratifying to see the Journal adopted by the International Society for Mountain Medicine, and its trajectory is definitely upward as befits its topic.

Hopefully other adventures in high-altitude physiology and medicine will come my way but even if they do not I have had more than my share and am grateful for this.

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Chapter 3

EXERCISE INDUCED ARTERIAL HYPOXEMIA: THE ROLE OF VENTILATION-PERFUSION INEQUALITY AND PULMONARY DIFFUSION LIMITATION

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Abstract: Many apparently healthy individuals experience pulmonary gas exchange limitations during exercise, and the term "exercise induced arterial hypoxemia" (EIAH) has been used to describe the increase in alveolar-arterial difference for oxygen ($AaDO_2$), which combined with a minimal alveolar hyperventilatory response, results in a reduction in arterial PO_2. Despite more than two decades of research, the mechanisms of pulmonary gas exchange limitations during exercise are still debated. Using data in 166 healthy normal subjects collated from several previously published studies it can be shown that ~20% of the variation in PaO_2 between individuals can be explained on the basis of variations in alveolar ventilation, whereas variations in $AaDO_2$ explain ~80%. Using multiple inert gas data the relative contributions of ventilation-perfusion ("$\dot{V}_A/\dot{Q}$") inequality and diffusion limitation to the $AaDO_2$ can be assessed. During maximal exercise, both in individuals with minimal ($AaDO_2$ < 20 Torr, x = 13±5, means ± SD, n = 35) and moderate to severe ($AaDO_2$= 25-40 Torr, x = 33±6, n = 20) gas exchange limitations, $\dot{V}_A/\dot{Q}$ inequality is an important contributor to the $AaDO_2$. However, in subjects with minimal gas exchange impairment, $\dot{V}_A/\dot{Q}$ inequality accounts for virtually all of the $AaDO_2$ (12±6 Torr), whereas in subjects with moderate to severe gas exchange impairment it accounts for less than 50% of the $AaDO_2$ (15±6 Torr). Using this framework, the difficulties associated with unraveling the mechanisms of pulmonary gas exchange limitations during exercise are explored, and current data discussed.

Key Words: multiple inert gas elimination technique, pulmonary gas exchange, perfusion heterogeneity

Hypoxia and Exercise, edited by R.C. Roach *et al.*
Springer, New York, 2006.

INTRODUCTION

For many years researchers have been fascinated by the apparent paradox that some highly aerobically trained humans and animals experience pulmonary limitations to maximal exercise performance. These are of sufficient magnitude to cause a reduction in arterial partial pressure of oxygen (PaO_2) and saturation (5, 17, 42, 52). This condition, termed exercise induced arterial hypoxemia (EIAH), poses a potential limitation to maximal exercise performance. Evidence suggests that a consequence of EIAH is that even small amounts of EIAH have a significant detrimental effect on limiting O_2 transport and utilization during maximal exercise (16, 40). The reader is referred to an excellent review of the topic (8), which reviews the potential causes of EIAH in detail and provides the framework for classification of the severity of hypoxemia which is utilized here.

Definitions of EIAH

In the past EIAH was defined in terms of a decrement in PaO_2 from resting levels, but this definition does not allow hypoxemia related to inadequate ventilation, to be distinguished from that due to from inefficient gas exchange. For example, a subject could have a increased alveolar-arterial difference for oxygen ($AaDO_2$) to 40 Torr, but if they were able to reduce markedly hyperventilate, and reduce the partial pressure of carbon dioxide to 25 Torr, PaO_2 would be maintained near resting levels. Thus, the effect of inefficient gas exchange would be obscured by a brisk hyperventilatory response. The most appropriate classification of the severity of EIAH therefore, depends on the type of research question is being asked. Where one is concerned with issues related to systemic oxygen transport and delivery, then arterial oxygen saturation (SaO_2) is the best indicator of the consequences of EIAH. If the main research question is concerned with efficiency of gas exchange, then a classification based on the extent of the increase of the $AaDO_2$ with exercise is more appropriate. An $AaDO_2$ greater than 25 Torr is consistent with a mild gas exchange impairment, whereas $AaDO_2$ greater than 35-40 Torr consistent with a severe gas exchange impairment (7). Similarly, when the hyperventilatory response to exercise is considered, a $PaCO_2$ at maximal exercise in the 35-38 Torr range represents a borderline hyperventilatory response and $PaCO_2$ greater than 38 Torr, an absent hyperventilatory response (7). The use of these different criteria allow the identification of key components of EIAH, which individually may not result in a decrement in PaO_2 or SaO_2. It should be noted that EIAH is not confined to humans and is especially notable in the horse which develops a large $AaDO_2$ during maximal exercise, associated with a considerable decline in PaO_2 (to ~70 Torr) and SaO_2 (~88%) (8, 52).

Why don't we know more about EIAH?

Despite considerable research effort, the causes of EIAH are still debated. Once issue is that the amount of data collected in healthy normal exercising subjects is rather small compared to the clinical data collected in patients with disease. In research studies, because of the technical difficulties associated with data collection, many studies are conducted using 8-12 subjects and generalizations to populations are therefore limited. Also, many of the desirable measurements can only be made indirectly, such as the quantification of pulmonary diffusion limitation using the multiple inert gas elimination technique (49). Many

investigators have chosen not to measure body temperature during exercise studies where arterial blood gas data is collected, since measuring temperature accurately often requires unpleasant and/or invasive monitoring. Since blood gases are measured at a standard electrode temperature of 37°C the true PaO_2 is underestimated, the $PaCO_2$ is overestimated. The use of pulse oximeters to estimate oxyhemoglobin saturation has induced a negative bias to the data such that the incidence of EIAH is greatly overestimated amongst populations of subjects (57). In the discussion that follows, temperature corrected arterial blood gas and inert gas data obtained in healthy normal subjects have been assembled from published sources (3, 5, 11, 13, 14, 22, 25, 28, 36, 39, 42, 51, 57), and used to discuss mechanisms of EIAH with special reference to ventilation-perfusion inequality and pulmonary diffusion limitation of oxygen transport.

Who gets EIAH?

Figure 1 shows the increase in the $AaDO_2$ and associated fall in PaO_2 from 198 exercise tests in healthy normal subjects exercising at 90-100% of $\dot{V}O_2$ max (data from 58 female, 123 male exercise tests, a total of 181 research subjects). The aerobic capacity of these subjects spans the physiologic range of $\dot{V}O_2$ from 30 to ~ 80 ml/kg/min. There is a negative relationship between $\dot{V}O_2$ max and PaO_2 at $\dot{V}O_2$ max, such that the subjects that have the highest $\dot{V}O_2$ max experience the greatest fall in PaO_2. However the PaO_2 during heavy and maximal exercise is highly variable even among populations of similar aerobic fitness. For example, at an oxygen consumption of 70 ml/kg/min the PaO_2 varies from 110 to 53 Torr. Even at more modest levels of $\dot{V}O_2$ max, there is a similar range of responses and although EIAH is often most pronounced at maximal exercise, there is a clear trend among some subjects towards developing a reduction in PaO_2 even during moderate exercise (17).

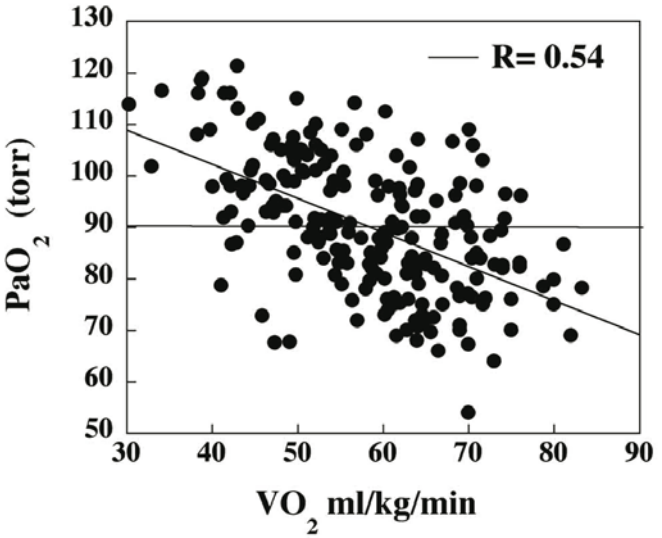

Figure 1. Arterial partial pressure of oxygen (PaO_2) obtained during cycling or treadmill running exercise at 90-100% of $\dot{V}O_2$max as a function of $\dot{V}O_2$. Data are from 181 exercise tests in 58 women and 123 men (some subjects were tested more than once). PaO_2 is reduced in subjects with higher $\dot{V}O_2$, although there is marked individual variability.

The gas exchange response to different exercise types varies even among the same subjects exercising at the same absolute and relative oxygen consumption (12, 23). For example, there is an approximately 10% lower PaO_2 during treadmill running than during

cycling exercise (23). Although it might be tempting to attribute the reduction in PaO_2 with running exercise to differences in alveolar ventilation, (and indeed $PaCO_2$ is greater during running than cycling) the efficiency of gas exchange also differs between the two exercise types and the $AaDO_2$ is greater during running (23), for reasons that are obscure. Women have significantly smaller lung volumes and a lower resting diffusing capacity for carbon monoxide than males, even when corrected for body size and lower hemoglobin levels (35, 46, 48). How these differences in pulmonary structure may manifest themselves functionally as EIAH has been recently reviewed (26).

MECHANISM(S) OF EIAH

What is the role of inadequate hyperventilation?

Inadequate hyperventilation during high intensity exercise is implicated as a contributor to EIAH (5, 18, 32, 41). However the extent of hyperventilation is highly variable between individuals. Figure 2 shows the relationship between PaO_2 and $PaCO_2$ in the same subjects as Figure 1. Since, at a constant metabolic rate, alveolar ventilation is inversely proportional to alveolar partial pressure of CO_2, $PaCO_2$ is used as an indicator of alveolar ventilation. Of these subjects, only 12 (7%) exhibit an absent hyperventilatory response as previously defined, whereas 114 (~70%) of subjects have an adequate hyperventilatory response. Even in the subjects with an absent hyperventilatory response, the variation in PaO_2 is substantial, ranging from 71-106 Torr, and PaO_2 is only loosely associated with $PaCO_2$ ($R^2 = 0.2$). Perhaps this is not surprising, since if the $AaDO_2$ is not substantially increased, frank hypoventilation, with $PaCO_2$ is increased above normal resting values is rare, and thus PaO_2 will not be reduce by this mechanism. However, the effects of limited hyperventilation on PaO_2 are amplified when gas exchange efficiency is impaired and the $AaDO_2$ is increased.

A number of mechanisms for the failure of some subjects to lower $PaCO_2$ below 35 Torr during exercise are possible including: a decreased peripheral chemoreceptor function (18), respiratory muscle fatigue (37, 38) and mechanical constraints imposed on inspiratory and expiratory flow (31, 32). This last explanation is the most likely. In young fit subjects almost all of the maximal expiratory flow volume curve may be approached during exercise (31) and even when helium-oxygen mixtures are used to remove expiratory flow limitation EIAH is not completely abolished (32). In addition the metabolic demands of the respiratory muscles themselves, consuming up to 15% of the total oxygen consumption, may also contribute to EIAH (6). Also, although ventilatory muscle fatigue may not play a major role during short term maximal and very low intensity exercise, respiratory muscle fatigue has been demonstrated during high intensity exercise (30) where it may further contribute to relative hypoventilation.

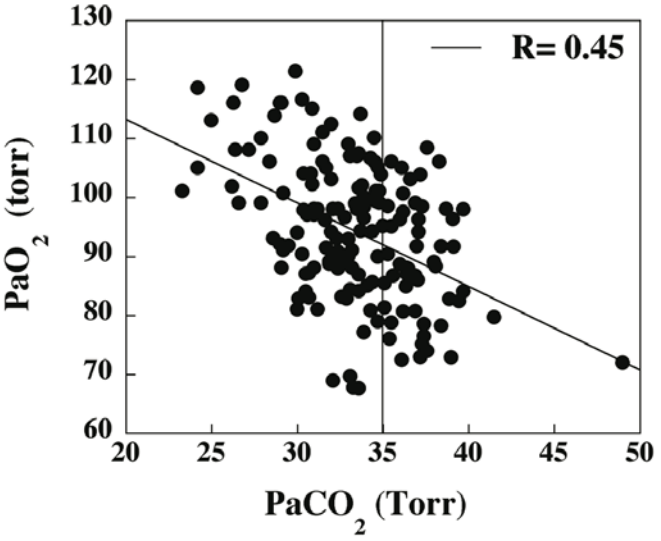

Figure 2. The relationship between PaO_2 and the partial pressure of carbon dioxide in arterial blood ($PaCO_2$, indicative of alveolar ventilation) from the same subjects and conditions as Figure 1. The majority of the subjects had an adequate hyperventilatory response as indicated by a $PaCO_2$ less than 35 torr, and frank hypoventilation is rare. There is a loose association between PaO_2 and $PaCO_2$, but marked inter-subject variability.

How important are differences in efficiency of gas exchange?

Figure 3 shows the $AaDO_2$ during near maximal or maximal exercise in 175 of the subjects from Figure 1. The variability in individual response is striking and the $AaDO_2$ varies almost 10 fold (~5-50 Torr) at any given level of $\dot{V}O_2$. While it can be appreciated that although most of the subjects who have an $AaDO_2$ greater than 25 Torr are exercising at a $\dot{V}O_2$ above 60 ml/kg min, there are still some who experience an $AaDO_2$ greater than 25 Torr at a $\dot{V}O_2$ of less than 50 ml/kg/min.

In determining any potential relationship between increased $AaDO_2$ and a decreased PaO_2 an interesting statistical problem arises. Regression analysis assumes that the X and Y variables are measured independently from one another. If the value of Y is used to calculate X, then these variables are said to be intertwined, and the regression analysis is invalid. Indeed, in these subjects, PaO_2 and $AaDO_2$ are highly related, and 85% of the variation in PaO_2 is explained on the basis of $AaDO_2$. Thus it is tempting to conclude that individual variation in gas exchange efficiency are important, however it is difficult to be sure because PaO_2 is used to calculate $AaDO_2$ and the variables are intertwined!

In Figure 4 subjects have been grouped according to the extent that the $AaDO_2$ is increased during maximal exercise, in order to compare subjects who experience a definite gas exchange limitation ($AaDO_2$ greater than 25 Torr, n = 69) to subjects that have minimal or no gas exchange limitation ($AaDO_2$ that is less than 20, n = 69). Figure 4A shows the $AaDO_2$ between the two groups. As expected, since they were grouped based on $AaDO_2$, the extent of gas exchange limitation is significantly greater in those subjects with an $AaDO_2$ greater than 25 and averages 33±6 Torr. That this difference in gas exchange efficiency has an effect on PaO_2 is not surprising and figure 4B shows the PaO_2 between the two groups. In the group with no gas exchange limitation, PaO_2 is maintained at normal resting levels, or is perhaps elevated slightly. However in the subjects with definite gas exchange limitations PaO_2 is reduced and averages 84 Torr.

Thus, it seems clear that gas exchange efficiency and an increased $AaDO_2$ is important in the development of EIAH. Potential mechanisms of reduced gas exchange efficiency with exercise and an increased $AaDO_2$ with exercise include intra-pulmonary or extra-pulmonary shunting, ventilation-perfusion $\dot{V}_A/\dot{Q}$ inequality, pulmonary diffusion limitation. A potential role of intrapulmonary shunts are discussed in Chapter 4.

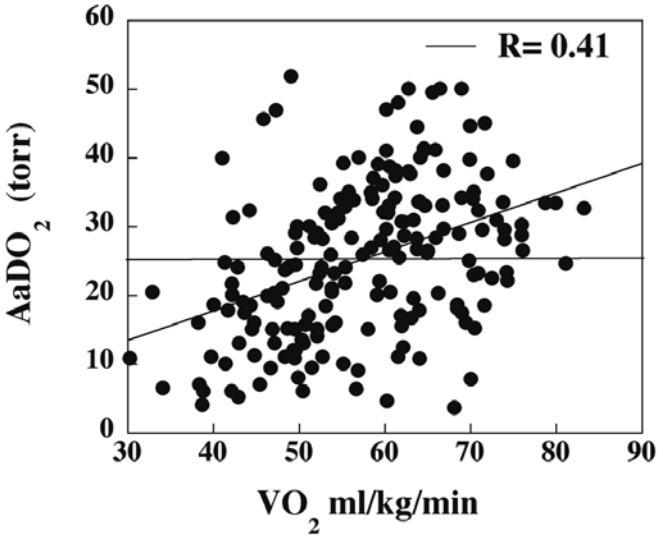

Figure 3. The relationship between alveolar arterial difference for oxygen ($AaDO_2$) and $\dot{V}O_2$ from the same subjects and conditions as Figure 1 and 2. Gas exchange is less efficient in subjects capable of higher oxygen consumptions, but at a $\dot{V}O_2$ of 70-80 ml/kg/min there is a 5-fold difference between the subjects with the greatest gas exchange limitations and the least.

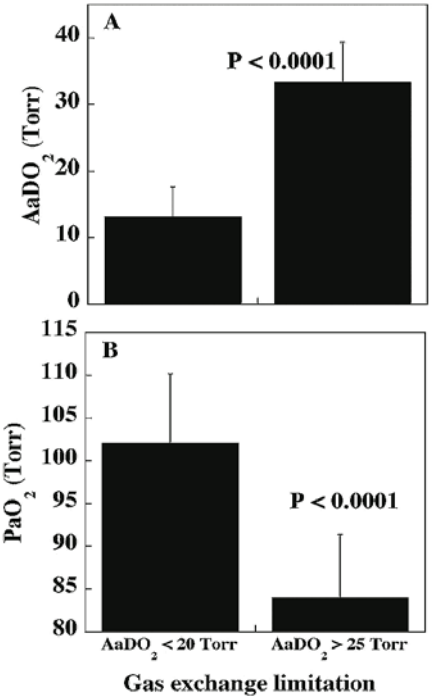

Figure 4. $AaDO_2$ data from a subset of the data presented in the preceding figures ($AaDO_2$ data were not available on all subjects). Subjects were grouped as having no significant gas exchange limitation ($AaDO_2$ at 90-100% of $\dot{V}O_2$ max <20 Torr, n = 69) or moderate to severe gas exchange limitation ($AaDO_2$ at 90-100% of $\dot{V}O_2$max > 25 Torr, n = 69). PaO_2 data from the same subjects as A. Subjects with no significant gas exchange limitation did not experience a fall in PaO2 at 90-100% of $\dot{V}O_2$max whereas subjects with moderate to severe gas exchange limitations experience a fall in PaO_2.

Multiple inert gas elimination technique

For decades, the multiple inert gas elimination technique has been used for measuring ventilation-perfusion ($\dot{V}_A/\dot{Q}$) inequality (53, 54). Despite the reliability and versatility of the multiple inert gas elimination technique, there are some significant drawbacks. It is time consuming and somewhat technically difficult to perform, it requires sampling of arterial blood and mixed expired gases, and it requires an independent measure of cardiac output, if pulmonary mixed venous blood is not sampled. This has limited the technique to only a few research centers in the world, despite more than 30 years of use.

Using inert gas data, the relative contributions of $\dot{V}_A/\dot{Q}$ inequality, diffusion limitation of oxygen transport, and intrapulmonary shunt to the $AaDO_2$ can be estimated (15). Trace amounts of six marker gases of differing solubility (sulfur hexafluorane (SF_6), cyclopropane, ethane, enflurane, ether, and acetone) that, unlike oxygen, have a linear relationship between solubility in blood and partial pressure are infused into a peripheral vein. Cardiac output and the concentration of the six gases in arterial blood and mixed expired gases are measured, and by using basic principals of mass balance, a distribution of pulmonary blood flow and ventilation relative to $\dot{V}_A/\dot{Q}$ ratio of the lung can be obtained. This method considers the lung "as if" it were comprised of fifty individual gas exchange units with different $\dot{V}_A/\dot{Q}$ ratios equally spaced on a logarithmic scale. Computer algorithms can be then be used to partition total ventilation and cardiac output between the fifty $\dot{V}_A/\dot{Q}$ compartments to minimize error between data predicted from the modeled $\dot{V}_A/\dot{Q}$ distributions and experimentally measured data (19). Using these distributions, a global index of $\dot{V}_A/\dot{Q}$ inequality can be calculated. The $LogSD\dot{V}$ and $LogSD\dot{Q}$. (the standard deviation of the ventilation and perfusion distributions, respectively) are used to express $\dot{V}_A/\dot{Q}$ inequality with larger number representing more $\dot{V}_A/\dot{Q}$ inequality (54).

Information is gained about intra-pulmonary shunts, and deadspace ventilation, since these form the extremes of ventilation-perfusion inequality -i.e. $\dot{V}_A/\dot{Q}$ is zero (shunt) or infinity (deadspace). In practical terms, blood flow to regions with a $\dot{V}_A/\dot{Q}$ ratio less than 0.005 is characterized as shunt, whereas ventilation to regions with a $\dot{V}_A/\dot{Q}$ ratio greater than 100 is characterized as deadspace ventilation.

To estimate diffusion limitation, the contribution that the measured amount of $\dot{V}_A/\dot{Q}$ inequality and intrapulmonary shunt makes to the $AaDO_2$ is calculated. Since inert gases are essentially invulnerable to diffusion limitation, anything not accounted for by $\dot{V}_A/\dot{Q}$ inequality can only come from diffusion limitation or extra-pulmonary non-cardiac shunts.

What is the role of ventilation-perfusion inequality in EIAH?

$\dot{V}_A/\dot{Q}$ inequality increases with increasing exercise intensity (11, 13, 28) but there does not appear to be a difference $\dot{V}_A/\dot{Q}$ inequality between highly fit subjects ($\dot{V}O_2max$ > 60 ml/kg/min) and those of low to average aerobic fitness ($\dot{V}O_2max$ < 50 ml/kg/min) (26). Although the amount of $\dot{V}_A/\dot{Q}$ inequality *within* an individual generally increases with increasing exercise intensity, there does not appear to be a significant relationship between the development of $\dot{V}_A/\dot{Q}$ inequality and aerobic fitness. Figure 5A shows the $LogSD\dot{Q}$. from inert gas data obtained in 55 subjects during exercise at 90-100% of $\dot{V}O_2max$ with and without pulmonary gas exchange limitation as previously defined. The two groups have similar $\dot{V}_A/\dot{Q}$ inequality at rest (data not shown) and the extent of the inequality is increased to a similar extent in both groups. The net effect on gas exchange is that $\dot{V}_A/\dot{Q}$ inequality

contributes approximate 12-15 Torr to the $AaDO_2$ (Figure 5A). In subjects without significant gas exchange limitations, this accounts for virtually all of the $AaDO_2$, however in subjects with significant gas exchange limitations it accounts for slightly less than 50% of the $AaDO_2$. That is not to say that $\dot{V}_A/\dot{Q}$ inequality is not important in the development of EIAH, but since subjects in both groups develop it to the same extent, it does not distinguish those who develop EIAH from those who do not. Since $\dot{V}_A/\dot{Q}$ inequality includes variations in alveolar PO_2 relative to blood flow, the potential effects of $\dot{V}_A/\dot{Q}$ inequality are in part mitigated by increased alveolar ventilation relative to perfusion which acts to shift the overall $\dot{V}_A/\dot{Q}$ distribution to the right. This can be though of as over-ventilation of area with limited perfusion (increased ventilation of area of high $\dot{V}_A/\dot{Q}$ ratio) rather than perfusion of area with low ventilation. The next effect is to minimize the effect of heterogeneity on gas exchange.

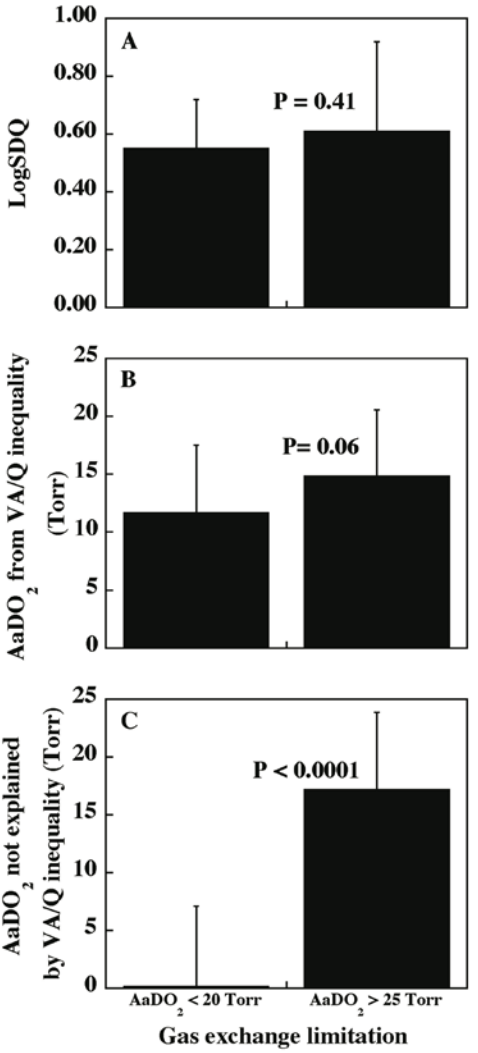

Figure 5. The contribution of $\dot{V}_A/\dot{Q}$ inequality, intra-pulmonary shunting and diffusion limitation to the $AaDO_2$ in 55 subjects in whom multiple inert gas elimination data was available in subjects with and without gas exchange limitation as previously defined. The increase in $\dot{V}_A/\dot{Q}$ in equality is similar between the two groups (A) and contribution of $\dot{V}_A/\dot{Q}$ in equality and intrapulmonary shunting to the $AaDO_2$ is also similar in both groups (B). However the subjects who have moderate or marked gas exchange limitations have an increased $AaDO_2$ not explained by inert gases (C) mostly likely due to pulmonary diffusion limitation of oxygen transport.

What are the causes of ventilation-perfusion inequality with exercise?

The physiological basis for increased $\dot{V}_A/\dot{Q}$ inequality with exercise is not certain, but a likely cause is interstitial pulmonary edema (8). Increased pulmonary vascular pressure with exercise reduce gravitational-dependent differences in blood flow at different heights in a lung but it also increases capillary filtration. Interstitial edema fluid would be expected to distort the surrounding architecture of the alveoli and capillary network. Altered airway and blood vessel diameter resulting from the presence of cuffing would affect distribution of blood flow, and perhaps air flow, in the lung. There is less evidence for increased heterogeneity of ventilation than for perfusion during exercise. However, a reduction of gas mixing in large airways during exercise would increase apparent $\dot{V}_A/\dot{Q}$ inequality measured by the inert gases (50).

Several lines of evidence implicate interstitial edema as the cause of increased $\dot{V}_A/\dot{Q}$ inequality (8). For example, $\dot{V}_A/\dot{Q}$ inequality is increased by hypoxia (11), and decreased by breathing 100% O_2, which would tend to increase and decrease, respectively, capillary pressure and filtration. Increased pulmonary artery pressure during exercise would also increase capillary filtration pressure and may have similar effects on the distribution of blood flow. Pigs, an animal that demonstrates increased $\dot{V}_A/\dot{Q}$ inequality with exercise (29) also show an increase in perivascular edema on histological examination in exercised animals, compared to resting controls (44). $\dot{V}_A/\dot{Q}$ inequality persists into recovery from heavy exercise, even after ventilation and cardiac output have returned to normal (45) and prolonged exercise results in a progressive increase in $\dot{V}_A/\dot{Q}$ inequality (25), consistent with fluid accumulation. Subjects who have a history of high altitude pulmonary edema (HAPE) also show larger increases in exercise induced $\dot{V}_A/\dot{Q}$ inequality compared to subjects without a history of HAPE (39). This has led to speculation that the underlying mechanism of increased $\dot{V}_A/\dot{Q}$ inequality with exercise and HAPE maybe the same.

Demonstrating that interstitial pulmonary edema occurs with exercise has been problematic, because many of the radiographic changes associated with edema are seen as a result of increased pulmonary blood flow, - a consequence of the increased cardiac output with exercise. Therefore to answer this question it becomes important to distinguish between intravascular and extravascular lung water. Acute clinical edema has been reported after ultra marathons, (33) and an increase in lung density is observed in subjects following a triathlon (4), and with exercise at moderate altitude (2), however this is not definite proof of the development of interstitial edema during sea level exercise. Recently, in a carefully conducted study, an increase in extravascular lung water, following sustained heavy exercise has been demonstrated using MRI (34). The MRI technique used has been validated against direct measures of water in isolated lung preparations with good results (10). These data demonstrate that interstitial edema does occur with exercise, but the corresponding effect on gas exchange remains to be demonstrated.

Is diffusion limitation of pulmonary oxygen transport important in EIAH?

As mentioned earlier, the extent of pulmonary diffusion limitation is inferred by estimating the contribution of $\dot{V}_A/\dot{Q}$ inequality and intrapulmonary shunt, measured from inert gas data to the $AaDO_2$ and attributing this to diffusion limitation. The problem with this

approach is that it is an indirect estimate of the extent of diffusion limitation, and is not able to differentiate the relative contributions of pulmonary O_2 diffusion limitation and extra-pulmonary shunting (e.g. from bronchial and thebesian veins) towards the overall A-aDO_2. Pulmonary shunting at rest via the bronchial and thebesian veins is believed to comprise <2% of total cardiac output (13, 49, 51), and is unlikely to change proportionally with exercise. Studies conducted during hypoxia indicate that such shunts would have to increase to 10-20% of cardiac output to explain the contribution to the $AaDO_2$ (42, 49). Since such shunts are anatomic, and will not change with FIO_2 it is unlikely that extra-pulmonary shunting contributes a more than a very small amount to the overall A-aDO_2, but this cannot be definitively stated.

The cause of the pulmonary diffusion impairment of oxygen transport is unknown, but is likely because of rapid pulmonary capillary transit of red blood cells (7). Few studies have attempted to measure transit times in exercising athletes and link the results to gas exchange. A significant fall in pulmonary transit time with exercise has been shown (24, 55, 58) but unequivocal evidence to show that transit times approached the hypothetical minimum time (24, 55) for oxygen equilibration is lacking. Thus pulmonary capillary transit time has not been established as a cause of pulmonary diffusion limitation. In part this is because the techniques used to measure pulmonary transit time either measured whole lung pulmonary transit of red blood cells, (however, capillary transit time is the important variable for gas exchange), or used inspired gases that alter the physiological conditions of measurement. Subjects who develop diffusion impairment often do so at submaximal levels of exercise (43) where presumably capillary recruitment is not maximal and transit time is not minimal, which also might argue against reduced transit times as being the cause. However it is the ratio of diffusional (D) to perfusional (βQ) conductance that determines the completeness of end capillary equilibration, (37, 38) and subjects with EIAH have a lower D/Q compared to those who do not develop EIAH (43). Thus it is possible that the perfusional conductance is recruited more quickly causing diffusion impairment even at relatively low levels of exercise.

Diffusion limitation from thickening of the blood-gas barrier is unlikely, but cannot be ruled out. After prolonged, heavy exercise in humans, the diffusing capacity for carbon monoxide decreases but there is no decrease in the O_2 diffusing capacity calculated from the inert gases (8). The decrease in diffusing capacity for carbon monoxide after exercise is best explained by a decrease in pulmonary capillary volume with a redistribution of central blood flow. There may also be a change in the matching of blood flow and diffusing capacity, which impacts gas exchange similar to $\dot{V}_A/\dot{Q}$ inequality (3).

What about shunts?

A shunt is defined as blood that enters the arterial system without coming in contact with ventilated areas of the lung (21, 56). Recently, two groups working independently have demonstrated the passage of bubbles through the pulmonary circulation during exercise using agitated saline contrast echocardiography (9, 47) and have suggested that this represent intrapulmonary shunts that affect gas exchange. These potential intrapulmonary shunts are controversial, because studies using inert gas data have never demonstrated significant shunts either at rest or during exercise (11, 13, 14, 25, 28, 39, 42, 49, 51). These bubble shunts observed during exercise are discussed in the next chapter and are briefly discussed here as they relate to inert gas data. Inert gases detect intra-pulmonary and car-

diac shunting because gases of low solubility such as SF_6 are ready exchanged even in regions of low $\dot{V}_A/\dot{Q}$, thus significant amounts in arterial blood signify right to left shunts. In fact, right to left shunts allow passage of all inert gases without excretion irrespective of solubility, and the overall picture is an increase in the retention of gases of all solubilities without an alteration in the excretion curve (20). It has been suggested that a reason for the discrepancy in findings between the two techniques is that pre-capillary gas exchange (in the vessels that allow the passage of microbubbles) of low solubility gases such as SF_6, underestimates intrapulmonary shunt (9, 47). This essentially means that that pre-capillary gas exchange would have to occur for SF_6 but not oxygen. While it is possible that pre-capillary gas exchange could affect the excretion of SF_6, other inert gases of low solubility such as such as cyclopropane, and ethane would also be affected, but to a lesser extent. This seems an unlikely explanation for the discrepancy, since these effects should be detected in other inert gases, but are not seen. Fluorescent microsphere data in pigs, and in horses (M. Hlastala-personal communication) do not demonstrate intrapulmonary shunts in these species, consistent with the inert gas data obtained, and in dogs when shunts are introduced experimentally, they are detected by inert gases (27). Also some patients with liver disease demonstrate positive intrapulmonary shunts with echocardiography, but have normal pulmonary gas exchange (1), and in patients that do have hypoxemia, shunts are also detected using the inert gas elimination technique (19). Thus it appears that the agitated saline contrast echo technique appears to be of limited ability to distinguish shunts that affect gas exchange. Perhaps limitations of the agitated saline contrast technique, (such as small diameter bubbles passing through normal capillaries, or deformation of larger bubbles such that bubbles traverse where red cells do not) may explain why bubbles appear to be shunted but red cell do not. Certainly further work needs to be done to clarify the role, if any these shunts play in gas exchange.

SUMMARY

Understanding the role of differing components of pulmonary gas exchange on the development of exercise induced arterial hypoxemia is far from complete. While 12-15 torr on average of the $AaDO_2$ is attributable to $\dot{V}_A/\dot{Q}$ inequality, it is the portion of the $AaDO_2$ that is not due to $\dot{V}_A/\dot{Q}$ inequality that distinguishes those subjects with marked pulmonary gas exchange limitations from those with minimal to none. There is a large body of evidence suggesting that interstitial pulmonary edema occurs as a result of exercise, which recent MRI data confirms. How this affects gas exchange has yet to be established.

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Chapter 4

INTRAPULMONARY SHUNT DURING NORMOXIC AND HYPOXIC EXERCISE IN HEALTHY HUMANS

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Abstract: This review presents evidence for the recruitment of intrapulmonary arteriovenous shunts (IPAVS) during exercise in normal healthy humans. Support for pre-capillary connections between the arterial and venous circulation in lungs of humans and animals have existed for over one-hundred years. Right-to-left physiological shunt has not been detected during exercise with gas exchange-dependent techniques. However, fundamental assumptions of these techniques may not allow for measurement of a small (1-3%) anatomical shunt, the magnitude of which would explain the entire A-aDO_2 typically observed during normoxic exercise. Data from contrast echocardiograph studies are presented demonstrating the development of IPAVS with exercise in 90% of subjects tested. Technetium-99m labeled macroaggregated albumin studies also found exercise IPAVS and calculated shunt to be ~2% at max exercise. These exercise IPAVS appear strongly related to the alveolar to arterial PO_2 difference, pulmonary blood flow and mean pulmonary artery pressure. Hypoxic exercise was found to induce IPAVS at lower workloads than during normoxic exercise in 50% of subjects, while all subjects continued to shunt during recovery from hypoxic exercise, but only three subjects demonstrated intrapulmonary shunt during recovery from normoxic exercise. We suggest that these previously under-appreciated intrapulmonary arteriovenous shunts develop during exercise, contributing to the impairment in gas exchange typically observed with exercise. Future work will better define the conditions for shunt recruitment as well as their physiologic consequence.

Key Words: pre-capillary gas exchange, hypoxia, intrapulmonary anastomoses, pulmonary circulation, arterial hypoxemia

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INTRODUCTION

For more than one hundred years, researchers have documented pre-capillary connections between the arterial and venous circulation (54). These arteriovenous anastomoses have been found in many anatomical locations associated with dynamic changes in blood flow including the skin, muscle, penis and the lung (8, 71). The existence of intrapulmonary arteriovenous anastomoses have been reported in infant (75) and adult humans (64, 66, 67), birds (45, 60), dogs (46), cats and rabbits (45) and in the gills of amphibian larvae (38). In several of these studies synthetic beads with diameters ranging from 50-390μm were able to pass through the pulmonary circulation (45, 64, 75). Although the indentity of the vessels responsible for transpulmonary passage of these synthetic beads remains undetermined, Tobin (64) identified vessels in 47% of the lobules of human lungs which were glomus-like and suggested that these vessels may act as intrapulmonary arteriovenous shunts (IPAVS). In addition, Elliot and Reid have described small muscular arteries in human lungs that branch from the conventional pulmonary arteries at right angles and are referred to as supernumerary arteries because they do not accompany airways (16). Interestingly, these supernumerary arteries have a muscular baffle at their origin which appears to regulate blood flow (57). Accordingly, these supernumerary vessels are an attractive candidate for being able to dynamically regulate pulmonary blood flow under hyperdynamic conditions such as exercise and hypoxia.

PREVIOUS WORK IN UNDERSTANDING GAS EXCHANGE INEFFICIENCY DURING EXERCISE

Our interest in these pulmonary arteriovenous anastomoses comes from a desire to understand gas exchange as it occurs in the lung. Pulmonary gas-exchange efficiency for oxygen is defined and quantified as the difference between the alveolar partial pressure of oxygen (PAO_2) and the arterial partial pressure of oxygen (PaO_2) and is known as the alveolar-to-arterial PO_2 difference ($A\text{-}aDO_2$). The normal $A\text{-}aDO_2$ in young, healthy individuals is 5-10 mmHg at rest and increases to 15-25 mmHg at maximal exercise (Figure 1). In some elite athletes the $A\text{-}aDO_2$ can widen as much as 25-40 mmHg, and depending on $A\text{-}aDO_2$ magnitude and the ventilatory response to exercise, exercise-induced arterial hypoxemia can develop (12). Approximately one half of the $A\text{-}aDO_2$ during exercise is said to be due to ventilation ($\dot{V}_A$) and perfusion ($\dot{Q}$) heterogeneity as measured by the multiple inert gas elimination technique (MIGET) (20, 65, 70), with the remainder due to extrapulmonary shunt, or diffusion limitation (70).

Extrapulmonary shunt results from the thebesian circulation, which empties directly into the left heart, and bronchial venus flow, which drains into the pulmonary vein. In humans, the O_2 content of the bronchial venous blood remains unknown. The contribution of these extrapulmonary shunts to arterial oxygenation would be expected to increase with exercise as mixed venous PO_2 falls (31). Although the total contribution of extrapulmonary shunt is assumed to be ~1% of the cardiac output (1, 2, 18, 19, 35, 40, 48), it should be considered an important contributor to the gas exchange inefficiency that occurs during exercise (see also anatomical shunt contribution calculations in (20)).

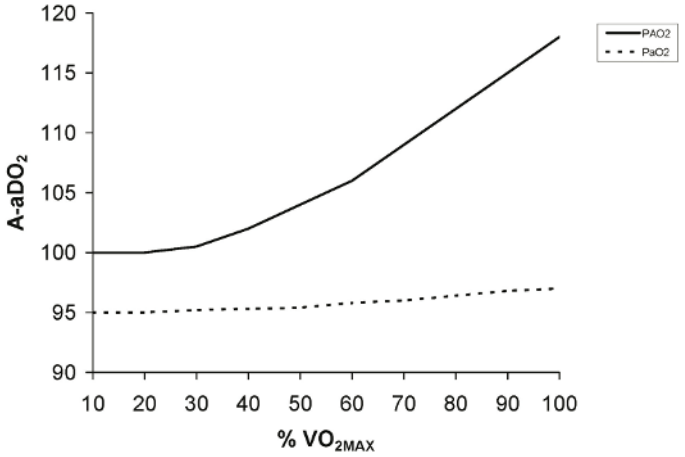

Figure 1. Alveolar PO_2 (PAO_2 - solid line) and arterial PO_2 (PaO_2 - dashed line) during progressive exercise to maximum in a healthy young adult. Despite a progressively widened A-aDO_2, arterial PO_2 is maintained near resting levels at all exercise intensities. Data compiled from the Rankin laboratory.

All previous studies using gas exchange-dependent methods have not documented the development of intrapulmonary shunting during exercise in healthy humans (12, 20, 23, 28, 51, 69). However, the absence of right-to-left intra-pulmonary shunt during exercise as measured by gas-dependent methods does not exclude the possibililty of exercise-induced IPAVS. Pulmonary pre-capillary gas exchange is well documented and the magnitude of pre-capillary gas exchange is critically dependent on the concentration gradient of the gas (10, 29, 30, 56, 59). Indeed Conhaim and Staub (10) have shown that pulmonary arteries can be oxygenated before reaching the alveolar capillaries, with the extent of the process dependent on time available for gas exchange and the PO_2 difference between alveolar gas and pulmonary arterial blood. For example, precapillary vessels up to 500µm are fully oxygenated when the lung is ventilated with 100% O_2. In light of evidence for pre-capillary gas exchange, Jameson suggested in 1964 that "The traditional view of the area in the pulmonary vascular bed from which diffusion occurs would appear to require revision."(30). Furthermore, in the case of the 100% oxygen technique, measurements of blood oxygen tension are not sufficiently accurate to distinguish shunts less than 10% of the cardiac output due to instrument error (50). As a result, Conhaim and Staub have stated that 100% O_2 breathing may underestimate arterial-venous shunts during normoxia. As pointed out recently, MIGET is based on early models of pulmonary gas exchange, which assumes. diffusion equilibrium between alveolar gas and end-capillary blood (26). Consequently, this technique may underestimate IPAVS because the detection of true shunt vs. low $\dot{V}_A\dot{Q}$ is dependent on the retention of any sulfur hexafluoride (SF_6), an inert gas with a very low solubility (and therefore a reduced retention) in blood (26). Accordingly, the low solubility and large concentration gradient between the blood vessels and airways may allow for pre-capillary elimination of SF_6. Recent work has reported that "…inert gases of all blood solubilities exchange in the airways…" and these authors went on to state that, "This effect complicates the interpretation of techniques that use highly soluble gases to evaluate lung function, such as MIGET"(56). As a consequence of precapillary gas exchange, the current gas exchange-dependent techniques used to determine right-to-left intrapulmonary shunt may underestimate or fail to detect IPAVS.

Intrapulmonary, or right to left, shunt can be caused by either low $\dot{V}_A/\dot{Q}$ regions secondary to atelectasis or alveolar flooding, or direct IPAVS. Pulmonary anastomoses as described above bypass capillaries, and therefore they should not participate in gas exchange at the capillary-alveoli interface. We hypothesized that the existence of any IPAVS during exercise would adversely affect exercise gas exchange, increasing the A-aDO_2. Indeed this right to left shunted blood is deoxygenated and would become increasingly so with exercise as oxygen extraction increases, reducing mixed venous PO_2. The magnitude of IPAVS would not need to be large during exercise, as Gledhill and associates previously calculated that an anatomical shunt of only ~2% of cardiac output would account for the entire A-aDO_2 of 17mmHg measured in their subjects during moderate intensity exercise (20). Therefore our studies examined if IPAVS developed during exercise, if these vessels are related to the widened A-aDO_2 during exercise, and whether these shunts could be modulated under different physiological conditions.

RECENT WORK IN UNDERSTANDING INTRAPULMONARY SHUNT & GAS EXCHANGE INEFFICIENCY DURING EXERCISE

We initially investigated the occurrence and prevalence of IPAVS during exercise in healthy humans using agitated saline contrast echocardiography, a non-gas exchange-dependent method (14). The validity of this standard clinical method for detecting cardiac and intrapulmonary shunting has been previously reported in detail elsewhere (7, 14, 39, 41, 42, 47, 52, 63). To detect shunt, sterile saline is agitated to create microbubbles that are injected into a peripheral vein. Contrast echocardiography distinguishes intracardiac shunting from intrapulmonary shunting, based on the number of cardiac cycles from appearance of contrast bubbles in the right heart to their appearance in the left heart. For example, if an individual has an intra-cardiac shunt due to a patent foramen ovale, contrast bubbles appear in the left heart immediately (< 3 cardiac cycles). However, if contrast bubbles appear in the left heart, after a delay (≥ 3 cardiac cycles), this is diagnostic of IPAVS (3, 21, 25, 34, 58). In these studies, agitated saline contrast echocardiograms were performed at rest and during progressive incremental cycle ergometer exercise to exhaustion. We enrolled 26 healthy active subjects. However, two subjects demonstrated atrial level shunting at rest and a third subject showed evidence of a previously unrecognized pulmonary arteriovenous malformation. These three subjects were excluded from the exercise studies. The remaining 23 healthy individuals (13♂ & 10♀, 23-48 years, mean $\dot{V}O_{2max}$ = 126% predicted) did not have evidence of shunting of contrast bubbles at rest (Figure 2). However, during exercise, ~90% of these subjects demonstrated a delayed appearance (>3 cardiac cycles) of saline contrast in the left heart which is indicative of IPAVS (Figure 2). Intrapulmonary shunting developed at submaximal exercise ($\dot{V}O_{2max}$ = 59±20 (SD)) and, once evident, persisted during all subsequent work rates. However, it appears that these shunts quickly closed following exercise, as no subject had positive contrast echocardiograms three minutes after termination of exercise (Figure 2). Fundamental limitations of this method include undetermined bubble diameter and therefore the inability to quantify shunt fraction or vessel diameter. While deformation of bubbles, and thus transpulmonary

passage, is possible, Roelandt found that a perfusion pressure of 300 Torr through a firmly wedged catheter was required to force any bubble contrast through the normal pulmonary circulation (52). This suggests that with normal physiological pressures, it is highly unlikely that bubble contrast would be able to traverse the normal pulmonary circulation in the absence of large diameter intrapulmonary shunts.

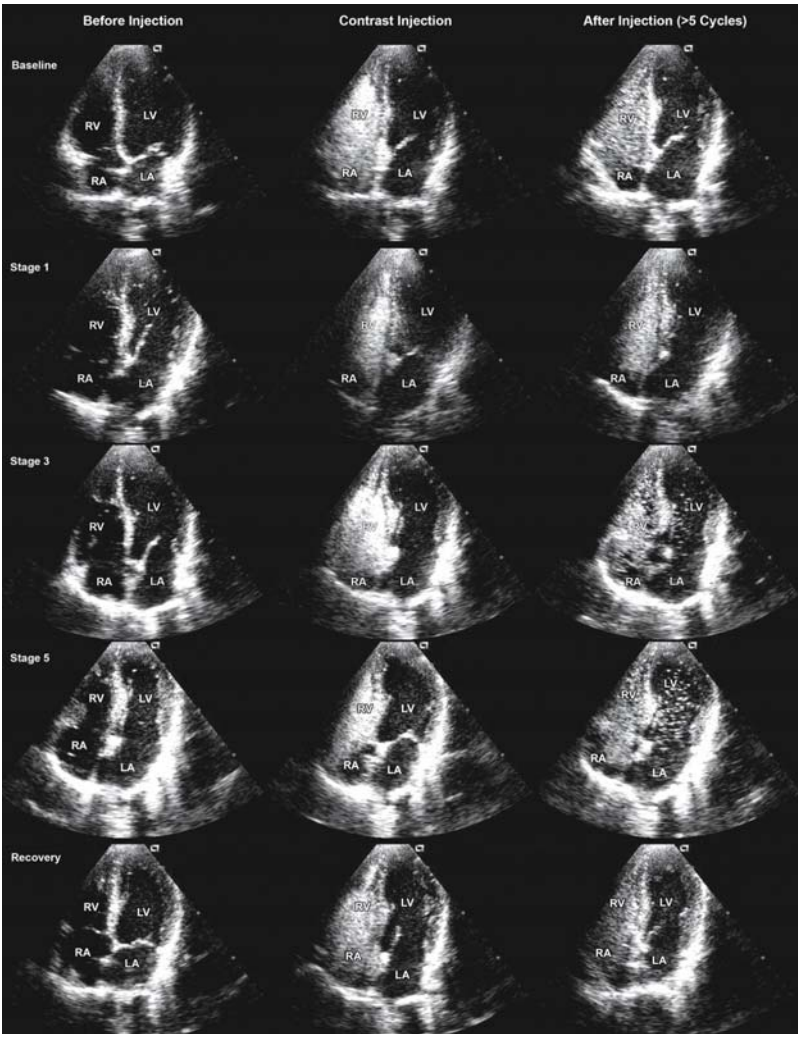

Figure 2. Exercise contrast echocardiograms at rest (Baseline) and 100, 200 and 260 watts (Stages 1, 3 & 5, respectively) in one subject. At rest (Baseline) and 100 watts (Stage 1), there is no evidence of intracardiac or intrapulmonary shunting, since the left heart is free if contrast bubbles. The first evidence of intrapulmonary shunting is seen at 200 watts (85% $\dot{V}O_{2max}$). Note the delayed appearance (>5cycles) of contrast bubbles in the left heart. The same pattern is seen at 260 watts. At three minutes post-exercise (Recovery), there is no evidence of intracardiac or intrapulmonary shunting, since the left heart is free if contrast bubbles. All images are apical 4-chamber views. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle.

Subsequent to our investigations using contrast echocardiography, we utilized a standard clinical nuclear medicine technique to determine the shunt fraction (36). This technique utilizes Technetium-99m labeled macroaggregated albumin (^{99m}TcMAA) and gamma camera imaging to detect and quantify shunt. This technique is similar to contrast echocardiography in that albumin aggregates larger than the diameter of the capillaries are filtered by the pulmonary microvasculature. Thus, if a large diameter (>20μm) vascular conduit opens up, the MAA particles will escape entrapment and become lodged in sytemic capillaries. This technique is superior to contrast echocardiography because the sizes of macroaggrgates are known - ninety percent of the MAA particles are between 10 and 90μm and the average size is 20 to 40μm with no particles greater than 150μm (6). Briefly, ^{99m}TcMAA was injected intravenously at rest, while standing on the treadmill, or during the last 30 seconds of a 3-minute maximal exercise bout. Rest and exercise studies were performed on different days, separated by at least 48 hrs. Within 5 minutes of injection, subjects underwent quantitative whole body imaging in the supine position. Anterior and posterior whole body images were obtained simultaneously using a dual-head gamma camera with low energy, high-resolution collimation. Shunt fractions were determined from geometric mean counts of anterior and posterior images, using a modified approach of Whyte and associates (74). Figure 3, shows the raw images generated from a male subject at rest and immediately after maximal treadmill exercise ($\dot{V}O_2$ = 3.3 L • min^{-1}). At rest, the majority of the ^{99m}Tc MAA particles are trapped in the lung, however following maximal exercise the increased signal in the leg muscles is indicative of ^{99m}Tc MAA particles passing through the lungs and being trapped in the muscle capillaries. It was determined that in healthy males (n=4), shunt fraction increased from a resting value of 0.46 ± 0.13% (mean ± SD) to 2.17 ± 0.78% at maximal exercise (P< 0.05). As noted previously (see above), a 2% shunt could account for all the impairment in pulmonary gas exchange (20) . Using this technique we were able to confirm our previous contrast echocardiography data that intrapulmonary shunts develop during exercise. We cannot rule out the possibility that some smaller particles (<20μm) may be able to traverse the pulmonary circulation via dilated capillaries at maximal exercise. However, in a study by Whyte et al., microspheres with a particle range of 7-25μm were used to investigate intrapulmonary shunting at rest and during submaximal exercise in healthy humans (74). The authors reported a shunt fraction of ~3% at rest rising to ~5% with submaximal (50% $\dot{V}O_{2max}$) exercise. The larger than expected shunt fraction at rest suggests that some of these microspheres could be passing through normal pulmonary capillaries with diameters ranging between 7-10μm (72). However, if the pulmonary capillaries were dilating substantially during exercise, then the calculated shunt fraction would be expected to surpass the reported 5%, suggesting limited pulmonary capillary dilation with submaximal exercise. Thus, despite these limitations, we believe that our findings using ^{99m}Tc MAA and gamma camera imaging provides further evidence for IPAVS during exercise.

Recently a separate laboratory has found that the recruitment of anatomical intrapulmonary shunts during exercise is associated with a widening of the A-aDO_2 (63). In this study by Stickland et al. (63), presence of intrapulmonary shunting was also determined with the agitated saline technique. Arterial blood gases were obtained to calculate A-aDO_2, while a Swan-Ganz catheter was inserted to measure pulmonary vascular pressure and allow for determination of cardiac output by direct Fick. In the upright resting position, A-aDO_2 was low (< 2.0 mmHg) while no subject demonstrated intrapulmonary shunt. With incremental

exercise there was a progressive widening of the A-aDO_2 in most subjects, while seven of eight subjects showed evidence of IPAVS during exercise (see Figures 4 and 5). Intrapulmonary shunting of contrast bubbles always occurred when A-aDO_2 exceeded 12 mmHg and cardiac output was greater than 24 L • min^{-1}. In the seven subjects who developed shunt, mean within-subject correlations showed that IPAVS development was related to A-aDO_2 (r = 0.68), cardiac output (r = 0.76), and mean pulmonary artery pressure (r = 0.73). Of note, the subject who did not develop IPAVS had the lowest $\dot{V}O_{2MAX}$, cardiac output and A-aDO_2 during exercise yet very high pulmonary vascular pressures. These results strongly suggest that the IPAVS, as assessed by contrast echocardiography, are related to the exercise-induced impairment in gas exchange.

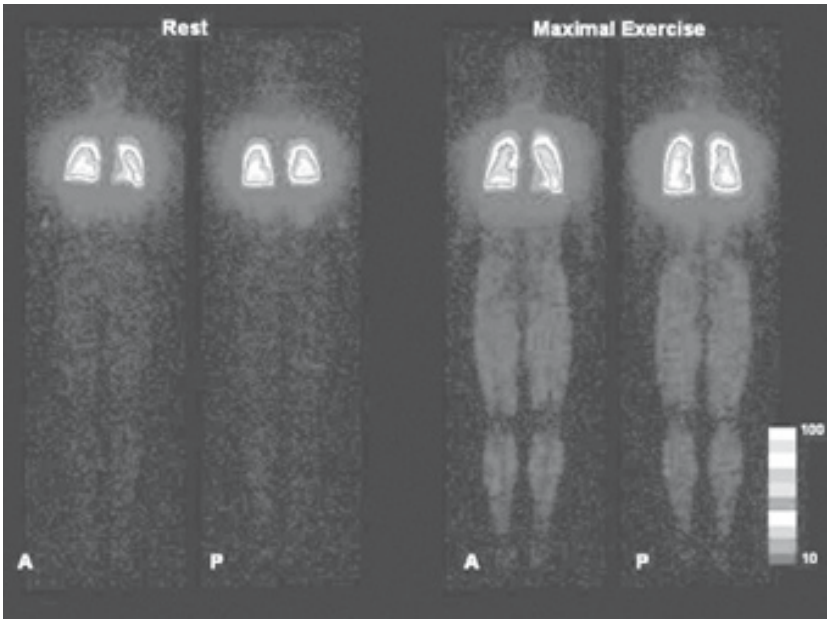

Figure 3. Anterior (A) and posterior (P) planar images obtained for rest and maximal treadmill exercise following injections with Technetium 99m-labeled macroaggregated albumin (^{99m}TcMAA). The increased number of counts in the exercising muscles (legs) indicates the transpulmonary passage of ^{99m}TcMAA. The shunt fraction in this individual at rest was 0.52% which increased to 2.83% at maximal exercise. Color bar represents increasing count intensities with lighter colors.

Next, we examined whether exercise-induced intrapulmonary shunting could be modulated with hypoxia (FiO_2=0.12). It was hypothesized that hypoxia would cause IPAVS at a lower workload than during normoxia because of the increased pulmonary vascular pressures and cardiac output that occur in response to hypoxic exercise (15). In this study, arterial lines were placed in 8 healthy males to examine the relationship between IPAVS and A-aDO_2 during hypoxic and normoxic exercise. No individuals shunted at rest during normoxia, however hypoxia caused shunting to occur at rest in 2/8 individuals. Seven of eight subjects shunted with normoxic exercise whereas all subjects shunted with hypoxic exercise (Figure 6). Unexpectedly, all subjects continued to shunt after the hypoxic exercise bout but only three of the subjects demonstrated intrapulmonary shunting of contrast

bubbles after normoxic exercise. Interestingly, one subject did not demonstrate intrapulmonary shunting during normoxic exercise and this subject's A-aDO_2 did not exceed 10 Torr (Figure 6) which is similar to the findings of Stickland et al. (63). See also arrow in Figure 4.

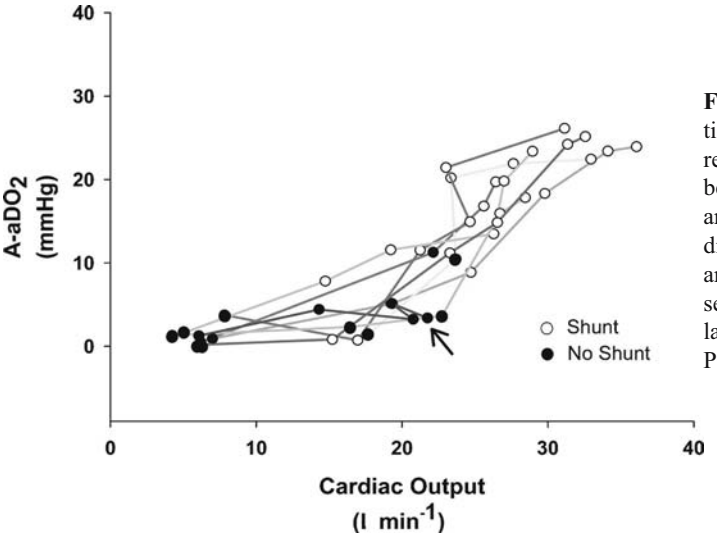

Figure 4. Individual relationships during upright rest and graded exercise between cardiac output and alveolar-arterial PO_2 difference upright at rest and during graded exercise. Note: A-aDO_2: alveolar-arterial PO_2difference.

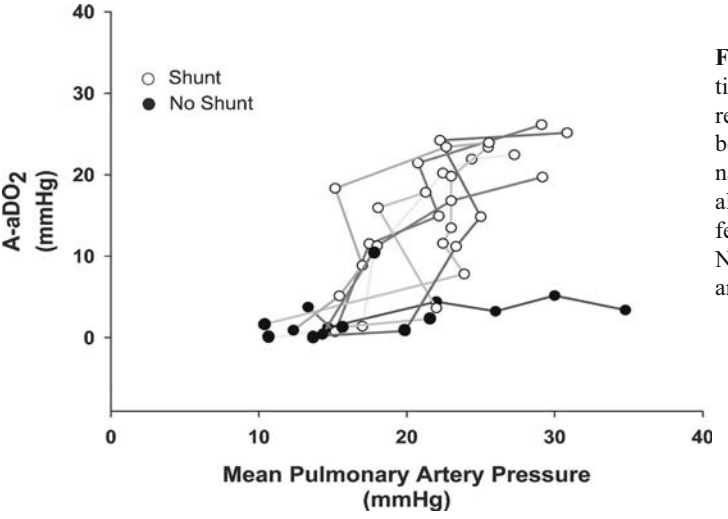

Figure 5. Individual relationships during upright rest and graded exercise between mean pulmonary artery pressure and alveolar-arterial PO_2 difference.
Note: A-aDO_2: alveolar-arterial PO_2 difference.

OTHER CONSEQUENCES OF IPAVS

In addition to the contribution to gas exchange, the recruitment of IPAVS may help to reduce pulmonary vascular pressures and right ventricular afterload during exercise. Indeed Berk *et al.* (4) first suggested that shunts may act as "pop-off valves" in response to increases in flow and pulmonary vascular resistance. The recruitment of these large diameter vessels would contribute to the reduction in pulmonary vascular pressure typically observed during exercise, limiting the driving pressure needed to maintain flow (49, 63). Based on the level of gas exchange impairment and our recent ^{99m}Tc MAA data, the total amount of blood flow shunted would have a small net effect on pulmonary artery pressure. However, MIGET has demonstrated that perfusion becomes increasingly heterogeneous with exercise (23, 44, 55, 65, 68, 70). IPAVS may be of critical importance in regulating microvascular pressures in the heterogeneously perfused lung whereby small local modulations in flow or pressure may prevent pulmonary edema or damage. The IPAVS which developed at a lower intensity during hypoxic exercise may thus serve as a protective mechanism to reduce local vascular pressures. For example, individuals prone to high altitude pulmonary edema (HAPE) appear to have a unique pulmonary vascular response to exercise with an exaggerated pulmonary vascular reactivity, higher pulmonary vascular resistances and greater exercise-induced $\dot{V}_A/\dot{Q}$ mismatch (15, 44). Approximately 10% of the subjects we have studied do not open intrapulmonary shunts during exercise. Are people who do not shunt during hyperdynamic states (e.g., exercise and exercise at altitude) more prone to pulmonary microvascular injury and thus exercise-induced alveolar hemorrhage and/or HAPE? Indeed, local recruitment of intrapulmonary shunts as regional pulmonary vascular pressures and flow increases would help divert the potentially damaging hydraulic energy away from vulnerable alveolar capillaries. It remains to be determined if these IPAVS are beneficial to the pulmonary vasculature or work capacity either under normal conditions, or extreme situations such as severe hypoxia.

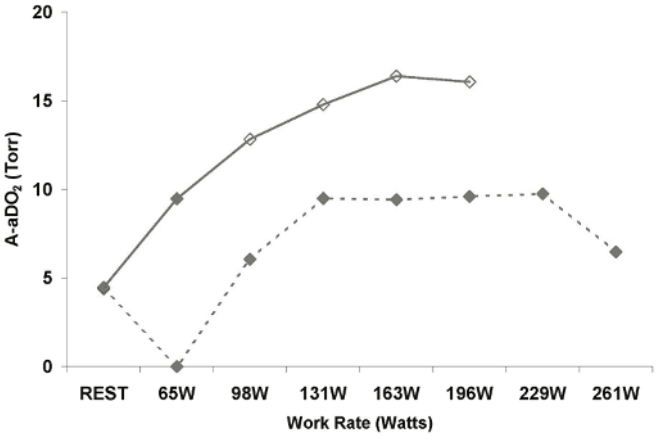

Figure 6. A-aDO$_2$ plotted against work rate in normoxia (red dashed line) and hypoxia (blue solid line). Filled symbol denotes no shunting, open symbol indicates right-to-left intrapulmonary shunting. Note than in normoxia, this subject did not shunt and the A-aDO$_2$ did not exceed 10 Torr. In hypoxia, this subject shunted at a lower workload and shunting inset occurred when the A-aDO$_2$ exceeded 10 Torr.

In addition to gas exchange, the lungs play an important protective role as blood filters. The pulmonary vasculature is generally regarded as very effective in preventing thrombi, platelet aggregates, and various other emboli from entering the systemic arterial circulation, resulting in embolic ischemic injury to the brain and heart. Indeed opening of intrapulmonary arteriovenous shunts during exertion or other hyperdynamic conditions may be important in ischemic stroke and heart disease. Indeed, even after extensive investigation 30-40% of ischemic stroke is considered cryptogenic, that is no clearly identifiable pathogenesis (24, 53). Furthermore, cryptogenic stroke is more common among young people (5, 17, 61, 62) and often associated with exertion (32). Paradoxical embolization has become an increasingly recognized cause of cryptogenic stroke (13) particularly in younger individuals without vascular risk factors (33). Paradoxical embolization occurs when emboli originating in the venous circulation bypass the normal filtering system of the pulmonary capillaries, enter the arterial circulation, and occlude arteries in various organs. Abnormal cardiovascular channels that may increase the risk of paradoxical embolization include patent foramen ovale (PFO), congenital cardiac defects, patent ductus arteriosus, and pulmonary arteriovenous malformations (PAVM). Numerous studies have found that cryptogenic stroke patients have an increased prevalence of PFO compared with patients with stroke of determined origin or normal control subjects (13, 33), and a meta-analysis found this increase to be most significant in younger patients (43). Even after surgical or percutaneous device closure, recurrent strokes occur (27). Reasons for recurrent ischemic events after either surgical or percutaneous PFO closure remain unclear. Thrombus formation at the site of surgical or device closure could serve as the potential embolic source. Furthermore, incomplete closure of a PFO may lead to subsequent paradoxical embolization. The cause for initial or subsequent stroke may not be paradoxical embolization through the PFO. Another potential channel that may increase the risk of paradoxical embolization are the inducible intrapulmonary arteriovenous conduits, we have described in this review. Indeed, these shunts maybe be invisible during standard diagnostic conditions (resting-supine). Understanding the structural and functional regulation of these inducible intrapulmonary conduits may add significantly to our understanding of a wide spectrum of diseases including cryptogenic stroke and ischemic heart disease.

INTRA-CARDIAC SHUNTING

In combination, Eldridge et al. (14) and Stickland et al. (63) observed atrial level right-to-left shunting of saline contrast in otherwise healthy, active subjects. With the original samples of both studies combined, intra-cardiac shunting was either observed at rest or developed with exercise in 9% (3/35) of the subjects. This is below the estimated frequency of intra-cardiac shunts in the general population of 27% (22). Of note, gas-dependent techniques such as MIGET have never detected intra-cardiac shunts at rest and during exercise in healthy subjects (23, 44, 65, 70). A subject with a right to left intra-cardiac shunt would be expected to develop a marked impairment in gas exchange with exercise. The data from subjects with intra-cardiac shunts were excluded from both Eldridge et al. and Stickland et al., however Stickland and colleagues did complete the exercise protocol with their one subject and observed a greater proportional increase in A-aDO_2 in the intra-cardiac shunting subject when compared to the IPAVS subjects (unpublished observation). Pulmonary

physiologists and clinicians must be aware of the prevalence of intra-cardiac shunts, particularly when a large A-aDO_2 is observed in an otherwise healthy subject.

FUTURE DIRECTIONS

The recent work using contrast echocardiography and ^{99m}TcMAA, indicates strongly that IPAVS develop in healthy humans during exercise. However, these results raise several pertinent questions. For example, it is not known with certainty whether these microbubbles/albumin aggregates are traveling through distinct inducible anastomoses or dilated pulmonary capillaries. Furthermore, it is not known what effect, if any, lung volume and pulmonary pressures have on intrapulmonary shunting. To address these questions, we have opted to take a reductionist approach using an isolated heart-lung preparation from rats. In these studies, the trachea was cannulated and the lungs were inflated to 15 cm H_2O. The pulmonary artery and the left atrium were cannulated and pulmonary artery pressure was set at 20cm H_2O and left atrial pressure was set to atmospheric pressure. Lungs were perfused with phosphate-buffered saline (PBS) with 10% albumin and 250k-1.5 million fluorescent-labeled microspheres in PBS solution was continuously infused into the arterial inflow line. The entire venous outflow was collected for one minute after infusion and vacuum filtered. The filter was imaged using fluoroscopy and microspheres were counted. Preliminary results have found that a small fraction (<1%) of 10 and 15 μm microspheres, but no 30 μm microspheres, were able to traverse the pulmonary circulation of the rat under zone III conditions (37). These data suggest that some pulmonary microvessels are considerably larger than previously believed (9, 72, 73).

CHALLENGES AHEAD

Pulmonary gas exchange is not perfect at rest and this imperfection is exacerbated by exercise. The possible contributors to the gas exchange inefficiency during exercise include; $\dot{V}_A/\dot{Q}$ heterogeneity, diffusion limitation as well as intra- and extrapulmonary shunt. There is long standing evidence for pulmonary anastomoses and we suggest that these vessels may contribute to the exercise-induced impairment in gas exchange. The fact that these anastomoses exist in mammals (including humans) as well as amphibians and birds suggests a common origin. It is possible that these vessels serve no “real” purpose in the adult human and are only remnant vessels from a common evolutionary pathway. Alternately, these vessels may be remnant fetal vessel that allow for blood flow through the lungs that bypass traditional gas exchange units at a time in development when gas exchange is not necessary (75). In either case, these vessels exist, but for undetermined reasons, are not detected by all techniques used to assess gas exchange at rest or during exercise. Future investigations into this area of respiratory physiology should aim to bridge the gap between the non-gas exchange and gas exchange-dependent methodology so that a more complete understanding of the complexities of gas exchange and pulmonary circulation can be determined.

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Chapter 5

EXERCISE-INDUCED ARTERIAL HYPOXEMIA: CONSEQUENCES FOR LOCOMOTOR MUSCLE FATIGUE

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Abstract: Reductions in arterial O_2 saturation (-5 to -10 % SaO_2 < rest) occur over time during sustained heavy intensity exercise in a normoxic environment, due primarily to the effects of acid pH and increased temperature on the position of the HbO_2 dissociation curve. We prevented the desaturation via increased F_IO_2 (.23 to .29) and showed that exercise time to exhaustion was increased. We used supramaximal magnetic stimulation (1 – 100 Hz) of the femoral nerve to test for quadriceps fatigue. We used mildly hyperoxic inspirates (F_IO_2 .23 to .29) to prevent O_2 desaturation. We then compared the amount of quadriceps fatigue incurred following cycling exercise at SaO_2 98% vs. 91% with each trial carried out at equal exercise intensities (90% Max) and for equal durations. Preventing the normal exercise-induced O_2 desaturation prevented about one-half the amount of exercise-induced quadriceps fatigue; plasma lactate and effort perception were also reduced. We conclude that the normal exercise-induced O_2 desaturation during heavy intensity endurance exercise contributes significantly to exercise performance limitation in part because of its effect on locomotor muscle fatigue. These effects of EIAH were confirmed in mild environmental hypoxia (FIO_2 .17, SaO_2 88%) which significantly augmented the magnitude of exercise-induced quadriceps fatigue observed in normoxia.

Key Words: quadriceps fatigue, central fatigue, force:frequency

INTRODUCTION

Exercise-induced arterial hypoxemia (EIAH) is defined as a reduction in arterial O_2 saturation (SaO_2) and occurs for a variety of reasons. During short-term incremental exercise

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in some highly trained subjects arterial PO_2 may fall secondary to an excessively widened alveolar to arterial PO_2 difference and in the absence of significant hyperventilation (1). If this EIAH is prevented (via increased F_IO_2), $\dot{V}O_2max$ is increased (5). During constant load, high intensity cycling or running exercise sustained to the point of exhaustion, SaO_2 falls progressively over time due primarily to a time- (and intensity-) dependent metabolic acidosis and rising body temperature, which shifts the O_2 dissociation curve to the right (see Figure 1). In some highly fit subjects (especially during running exercise) a reduced PaO_2 will also contribute to a reduced SaO_2 (11) (see Figure 2). Preventing this desaturation by adding small amounts of hyperoxic inspired gas mixtures (.23 - .30 F_IO_2) induces an increase in exercise time to exhaustion (See Figure 3). Furthermore, if the O_2 desaturation is exacerbated by acutely reducing F_IO2 or ascending to high altitudes, exercise time to exhaustion is further reduced (Figure 3).

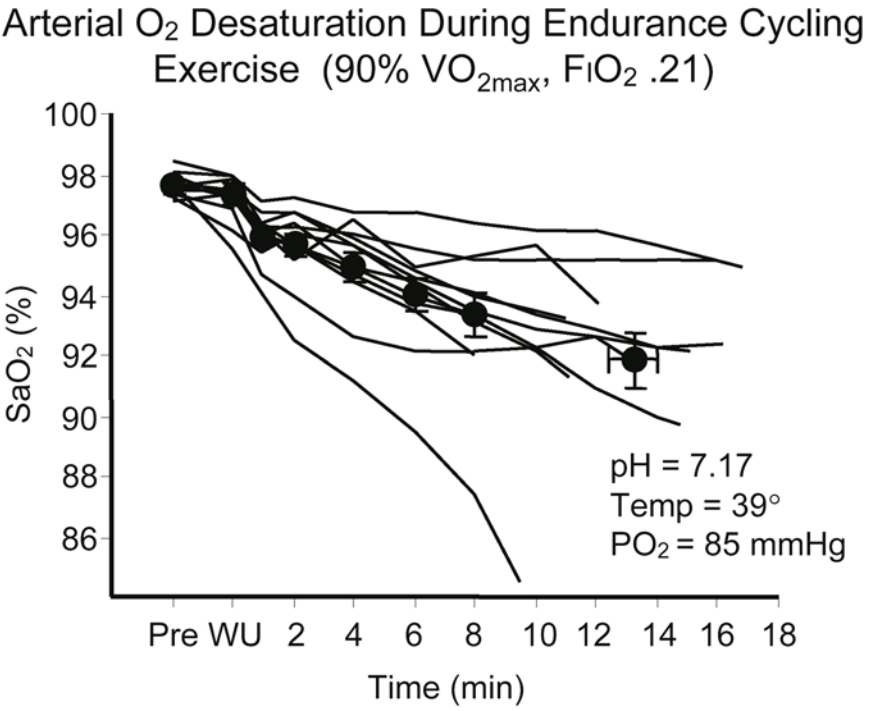

Figure 1. EIAH during heavy intensity, constant load cycling exercise in 11 fit young adult male cyclists. The O_2 desaturation was due primarily to a time-dependant metabolic acidosis (pH ~ 7.17) and rise in temperature (~ + 2°C) as PaO_2 was 80 – 90 mmHg.

HYPOTHESES

We asked the fundamental question, "Why does arterial hypoxemia—either the 6 to 10% reduction in SaO_2 induced by prolonged heavy exercise in a normoxic environment or the more severe O_2 desaturation encountered during prolonged heavy exercise at moderately high altitudes—curtail performance time?" Is this curtailment strictly a result of reduced O_2 transport to working locomotor muscle leading to "peripheral" end-organ fatigue? This

peripheral fatigue effect is certainly a reasonable hypothesis given the evidence that hypoxemia will reduce Ca2+ reuptake and release in the sarcoplasmic reticulum, thereby decreasing cross-bridge activation and force output (2); and this effect may occur through a number of mechanisms, including accumulation of lactate and hydrogen ions, inorganic phosphate and/or free radical production (3). Alternatively, the long-held concept of a "central governor" limiting motor recruitment of working muscle such that the function of vital organs is protected may explain exercise limitation in the presence of hypoxemia. Hence, this latter hypothesis would require reflex inhibition of central motor output to locomotor muscles in order to protect against vital organ system failure (7, 8).

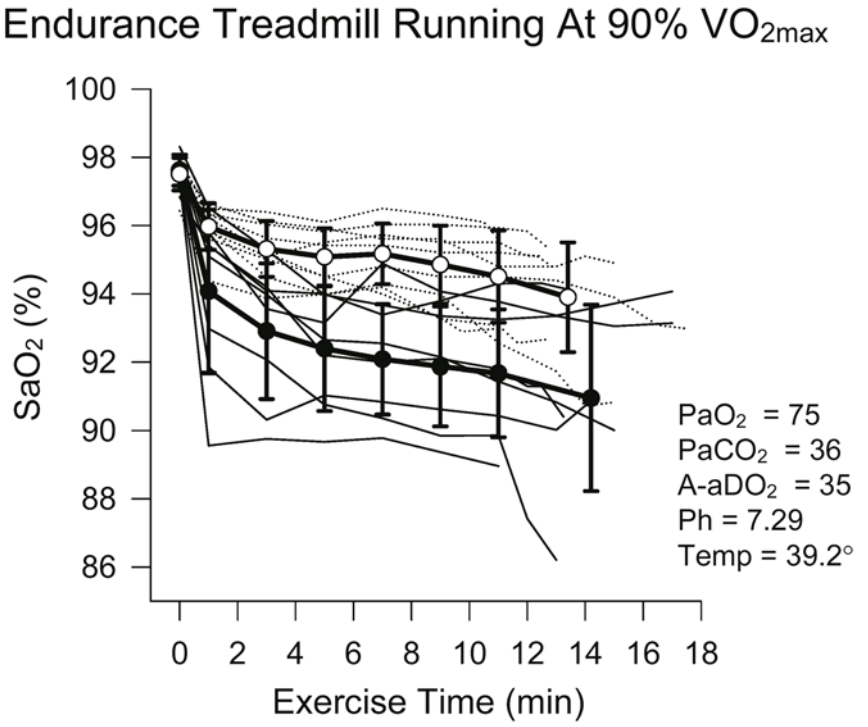

Figure 2. Arterial O_2 saturation during constant load, high intensity treadmill running to exhaustion in 17 fit young women (F_IO_2 .21). Mean values are shown for those with a low PaO_2 (~ 75 mmHg) (closed circles) and those who maintained a high PaO_2 (85 – 90 mmHg) (open circles) throughout. The $PaCO_2$ was higher and the A-aDO_2 wider in the low PaO_2 group. The time-dependant fall in SaO_2 beyond the first two minutes of running was due to the rise in temperature (+2.2°C) and fall in arterial pH (~ 7.25 pH; From Wetter et al. (11)).

Limiting the duration and/or magnitude of cerebral hypoxia may present yet another potential source of central inhibition of locomotor muscle recruitment. A recent study prevented EIAH during maximal rowing exercise and observed an increase in brain oxygenation but no change in muscle oxygenation, thereby implying that changing SaO_2 had little effect on muscle O_2 transport, per se (6). Indeed, the classic studies of John Sutton, Jack Reeves and colleagues in Operation Everest II predicted a major role for non-peripheral factors in limiting exercise performance during the simulated ascent of Everest (4, 9).

General Methods

We used supramaximal magnetic stimulation of the femoral nerve before and after cycling exercise to determine if indeed locomotor muscle fatigue, per se, was induced by changing levels of arterial oxygenation during high intensity exercise in normoxic and in hypoxic environments. This procedure consisted of paired, supra-maximal stimuli delivered over a range of frequencies (1 – 100 Hz), achieved by varying the duration of the interstimulus interval. The quadriceps force output in response to supramaximal nerve stimulation was shown to be highly reproducible (coefficient of variation < ± 6%) both within- and between-days. Evoked potentials in response to nerve stimulation were measured from the quadriceps muscle EMG; their magnitude remained unchanged from baseline to post exercise conditions ensuring that the motor input to the muscle was supramaximal and equal before and after the cycling exercise. Superimposition of a supramaximal twitch on a maximum voluntary quadriceps contraction produced an average 7% increase in force output, i.e., the voluntary effort produced a force that was 93% of truly maximal force output that was 7% of the potentiated twitch value at rest, indicating that subjects did not fully activate their quadriceps via voluntary effort under resting baseline conditions.

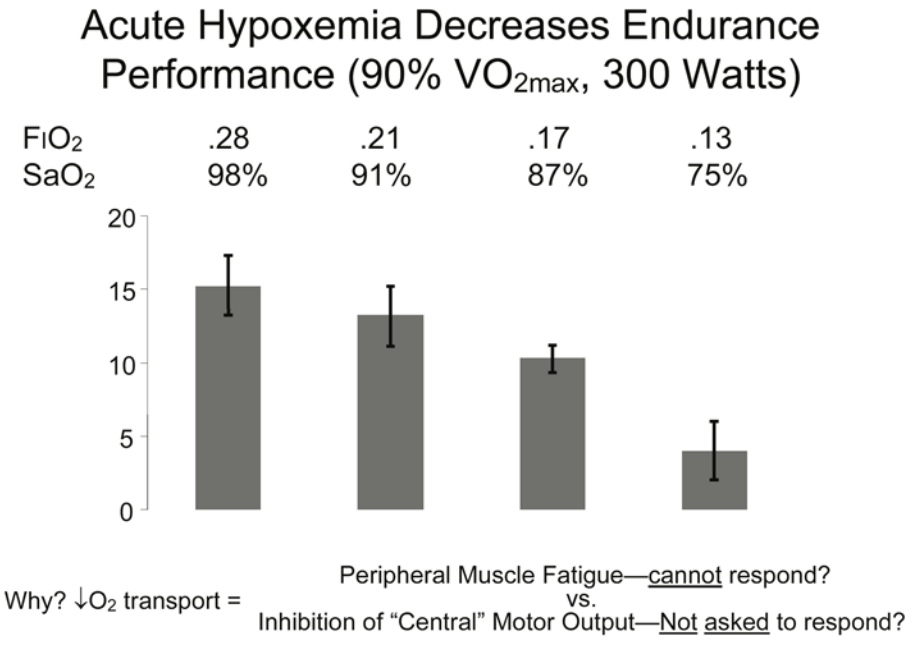

Figure 3. Effects of EIAH on time to exhaustion at a fixed, high intensity work rate. Note in a normoxic environment with an end-exercise arterial O_2 saturation which averaged 91% (see Fig. 2) time to exhaustion was about 13 minutes. Preventing this reduction in SaO_2 (via FIO_2 .23 to .29) allowed the subjects to exercise at least 16% longer, whereas reducing F_IO_2 below normoxic levels caused moderate (at F_IO_2 .17 and 87% SaO_2) and then marked (at .13 F_IO_2 and 75% SaO_2) reductions in exercise time to exhaustion.

EXPERIMENT A. PREVENTING EIAH IN A NORMOXIC ENVIRONMENT

Subjects cycled at a fixed workload at an intensity that averaged 90% of their peak maximal work rate, until they could no longer maintain a target pedaling frequency. Arterial blood was obtained periodically and magnetic stimulation was applied at baseline and at intervals from 2.5 to 70 minutes following exercise. Then the subjects returned and repeated the experiment only with supplemental inspired O_2 (.23 - .29 F_IO_2) added in amounts that were just sufficient to prevent EIAH, i.e., SaO_2 was maintained at resting levels (~98%). On this second day subjects exercised at power outputs and for durations that were identical to those under control (FIO2 .21) conditions. Thus the only difference between the two exercise conditions was the SaO2, i.e., 91% vs. 98%.

The key fatigue findings are summarized in Figure 4. Note that exercise in normoxia, which caused a progressive desaturation to 91% SaO_2 (range = 87 – 93%), resulted in a reduction of force output immediately following exercise at all stimulation frequencies (1 – 100 Hz) that averaged 33% below baseline and returned gradually to baseline levels over 70 minutes of recovery. When the EIAH was prevented and SaO_2 held at resting levels, the reduction in force output was still significant but only about one-half that which occurred under control conditions in the presence of EIAH. Thus, the prevention of EIAH, per se, significantly reduced the amount of quadriceps fatigue induced by the exercise; it also significantly lowered the absolute level and rate of rise of arterial blood lactate concentration over the final half of the exercise, and reduced the rate of rise of effort perception for both limb discomfort and dyspnea. Finally, using the twitch stimulation superimposed on the MVC, we observed that voluntary activation of the quadriceps was reduced from 93% at baseline to 85% following exercise in normoxia; and when desaturation was prevented, voluntary activation following exercise was increased to 90%.

These findings demonstrate that the arterial O_2 desaturation that normally accompanies heavy intensity sustained exercise in a normoxic environment contributes significantly to locomotor muscle fatigue. In turn, we think it reasonable to conclude that the lessening of local muscle fatigue with the prevention of O_2 desaturation contributes to an enhancement of exercise performance. While these data clearly implicate a significant effect of reduced O_2 transport on locomotor muscle fatigue and on exercise performance, they do not rule out an effect of O_2 desaturation on reducing motor output to the locomotor muscles during exercise i.e. “central fatigue.” Indeed the finding that exercise significantly reduced voluntary activation of the quadriceps, and that this was largely relieved by preventing O_2 desaturation, indirectly implicates a contribution from “central fatigue” to hypoxemic effects on exercise limitation. A major outstanding problem with interpretation of these tests is whether the change in force output between the voluntary isometric effort and the sum of the voluntary effort plus superimposed twitch, as conducted in the resting subject during recovery, truly represents “central inhibition” of the volitional force produced during the preceding rhythmic exercise task. To date there is no direct evidence—pro or con—of an effect of arterial hypoxemia on reflex inhibition of central motor output to locomotor muscles during exercise. Certainly the reduced rates of rise of effort perceptions during exercise when EIAH was prevented might also have contributed to exercise performance limitation and may be classified as “central” fatigue (or “symptom limited”). However since much of the cause of enhanced effort perceptions in the presence of hypoxemia likely

originated from intensified sensory feedback input from fatiguing, acidic muscles, then this type of "central" fatigue is causally linked to "peripheral" fatigue.

Nevertheless, we cannot claim a true cause: effect relationship, because we do not know how these data obtained during supramaximal nerve stimulation in recovery translate precisely into the subject's capability for sustaining a given (likely sub-maximal) power output during the preceding exercise. Indirect evidence of hypoxic effects on peripheral muscle fatigue during heavy intensity exercise is available from Taylor et al. (10) who found increases in integrated quadriceps EMG over time during exercise which were greater in severe hypoxia than in normoxia.

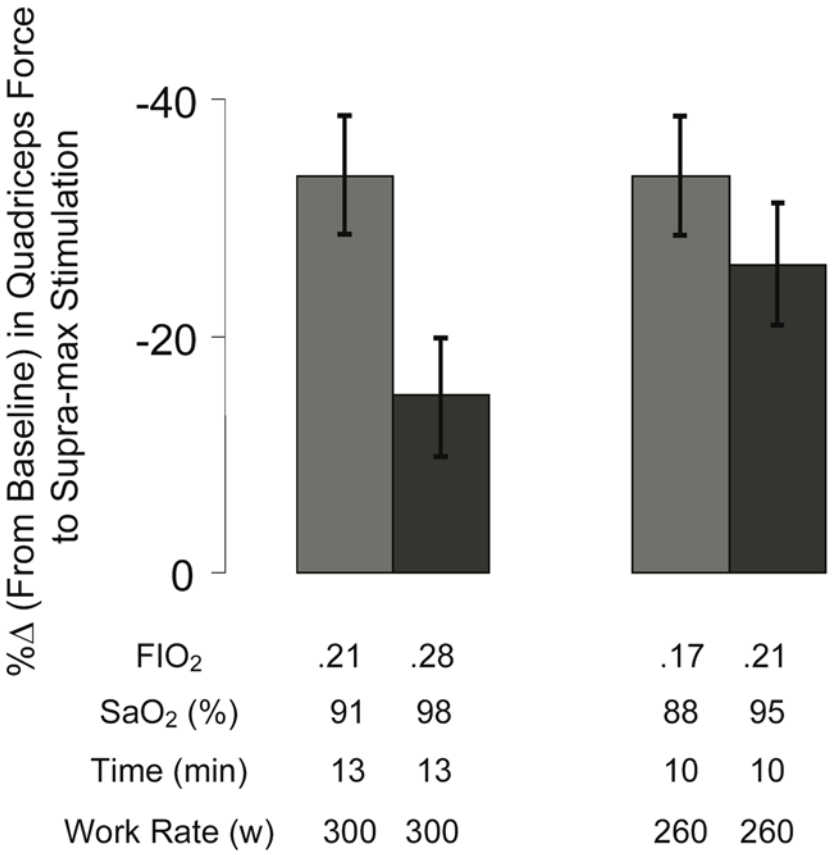

Figure 4. Cycling exercise to exhaustion in normoxia caused a reduction in force output of the quadriceps in response to supramaximal femoral nerve stimulation which averaged one-third below baseline. When the hypoxemia was prevented (.27 F_IO_2) and the exercise carried out for an identical time and work rate as at F_IO_2 .21, quadriceps fatigue was reduced by more than 50%. When EIAH was made greater by mild environmental hypoxia (F_IO_2 .17), quadriceps fatigue was enhanced.

EXPERIMENT B. EFFECT OF HYPOXIC-INDUCED MODERATE HYPOXEMIA

This experiment was conducted in those subjects who experienced minimal O_2 desaturation (~ 95%) during the exercise in normoxia. A similar design was used as in experiment A, in that the effect on quadriceps fatigue was compared following exercise of identical work rates and durations. In these subjects F_IO_2 of .17 reduced the mean exercise SaO_2 to 88% and significantly increased the amount of quadriceps fatigue by 20 – 25% over that observed at F_IO_2 .21 (SaO_2 95%) (see Figure 3). Furthermore the moderate reductions in SaO_2 below 90% increased the rate of rise of blood lactate and effort perceptions during the exercise. So again, as with the prevention of EIAH in a normoxic environment, the further reduced SaO_2 in a mildly hypoxic environment was linked to performance limitation by means of O_2 transport-induced reductions in the force output of the locomotor muscles in response to supramaximal motor nerve stimulation.

We propose that the effect of EIAH on locomotor muscle (peripheral) fatigue mechanisms were due to reductions in O_2 transport to muscle which in turn would reduce muscle capillary and mitochondrial PO_2. Since the exercise in our study required a work rate very close to $\dot{V}O_2$max, preventing the O_2 desaturation also raised mean $\dot{V}O_2$ about 5%, at end-exercise. Thus, subjects were exercising at a slightly lower relative work intensity which would account for at least some of the reduction in lactate production and fatigue.

SUMMARY

The schematic diagram in Figure 5 outlines the various types of contributions to curtailment of performance experienced in mild through moderate hypoxemia. Listed are peripheral muscle fatigue secondary to reduced O_2 transport to muscle and two types of "central" factors, namely conscious effort perception and reflex inhibition, which might limit performance by reducing motor output to the working locomotor muscles. Our results show that at all levels of hypoxemia, its effect on limiting performance time was consistently associated with significant hypoxemic-induced peripheral (i.e. locomotor muscle) fatigue. We especially emphasize that even in a normoxic (i.e. sea-level) environment, the 6 to 10% arterial O_2 desaturation normally produced during heavy intensity, sustained, exercise in healthy subjects is sufficient to significantly exacerbate locomotor muscle fatigue. An additional contribution to exercise limitation occurs from the two types of "central" influences inhibiting motor output to the limb muscles during exercise. One of these "central" factors, i.e. conscious effort perception, is strongly influenced by peripheral muscle fatigue, per se. The other, "reflex" inhibition, has not been measured directly during whole body exercise. A significant contribution from one or more of these "central" influences is likely to be present at all levels of arterial hypoxemia; and they may contribute substantially to exercise limitation in severe levels of hypoxemia.

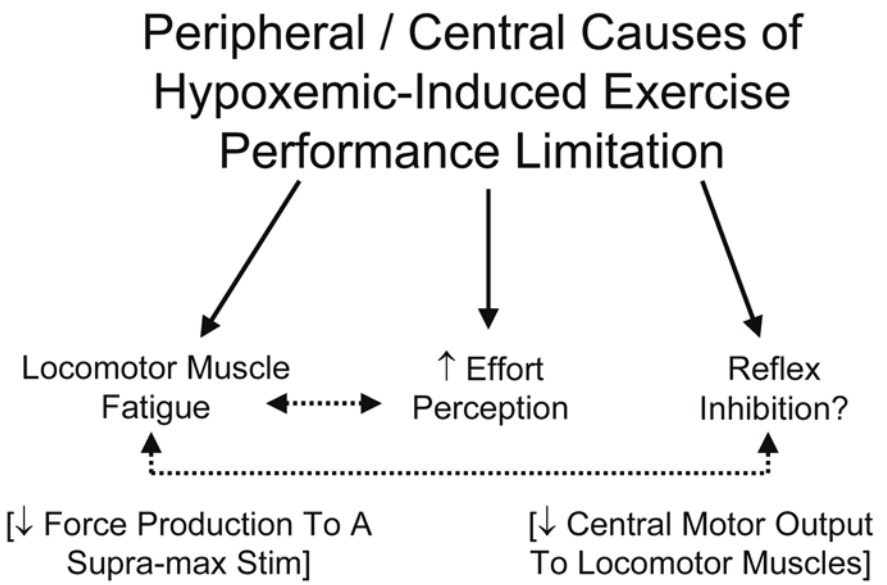

Figure 5. Schematic of the "peripheral" and "central" fatigue influences on limitations to exercise performance caused by exercise-induced arterial hypoxemia. We found peripheral locomotor muscle fatigue to be induced by all levels of arterial hypoxemia studied—including the EIAH which occurs during heavy sustained exercise in normoxia. Indirect evidence also implicates "central" fatigue contributions—especially in severe environmental hypoxia-induced arterial hypoxemia.

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Chapter 6

MECHANISMS OF SLEEP APNEA AT ALTITUDE

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Abstract: At altitude normal people often develop periodic breathing in sleep - regularly recurring periods of hyperpnea and apnea. This phenomenon is probably explained by instability of the negative feedback system for controlling ventilation. Such systems can be modeled by sets of differential equations that describe behavior of key components of the system and how they interact. Mathematical models of the breathing control system have increased in complexity and the accuracy with which they simulate human physiology. Recent papers by Zbigniew Topor et al. (5,6) describe a model with two separate feedback loops, one simulating peripheral and the other central chemoreceptor reflexes, as well as accurate representations of blood components, circulatory loops and brain blood flow. This model shows unstable breathing when one chemoreceptor loop has high gain while the other has low gain, but not when both have high gain. It also behaves in counter-intuitive way by becoming more stable when brain blood flow is reduced and unresponsive to blood. gas changes. Insights from such models may bring us closer to understanding high altitude periodic breathing.

Key Words: mathematical modeling, central apnea, unstable control systems, chemoreceptor feedback

INTRODUCTION

Apnea is one feature of "unstable breathing", an engineering term used to describe a pattern of breathing in which tidal volume gradually increases to maximum, then decreases to zero, where it remains during the apnea, then increases again in a recurring periodic pattern. At sea level, such recurrent apneas are typically 'obstructive'. The pharynx falls closed because of relaxation of pharyngeal dilator muscles in sleep. During the apnea, the diaphragm continues to contract but no air flows through the obstructed pharynx. CO_2 gradually rises and PO_2 falls until the chemical stimuli cause sufficient activation of pharyngeal dilator muscles to reopen the pharynx and permit breathing to resume. At altitude,

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or on exposure to low inspired oxygen, on the other hand, apneas are typically ?central? meaning that when tidal volume falls to zero there is no obstruction and no efforts are made by respiratory muscles during the apnea. (1)

The periodic breathing of altitude can be explained by engineering theories of negative feedback control systems. Such systems are considered unstable if they display a persistent pattern of periodic behavior in response to a disturbance in the controlled output.

A familiar example is the system that controls temperature in a shower. In the showerhead, water from a hot water source and water from a cold water source mix to produce water of a certain temperature. When the water falls on the skin of the person in the shower, temperature receptors signal the temperature to the central nervous system, which compares this temperature with the desired temperature and when necessary emits a command to correct the temperature. If the water temperature is too high, the command is sent through nerves to muscles of the hand and arm which cause the hand to reach out and turn the tap controlling the flow of hot water until the mixture of water coming from the showerhead is at the desired temperature. This is called a negative feedback system because an increase in temperature perceived at the temperature receptors leads to a decrease in the flow of hot water into the showerhead and thus to a corrective decrease in temperature.

The shower control system can be unstable if there is a long pipe between the tap that adjusts the flow of hot water and the showerhead. This causes a delay between the time the tap is adjusted and the time the resulting temperature is perceived at the temperature receptor. If somebody flushes a toilet nearby so that the flow of the cold water into the showerhead goes down and the temperature of the water striking the skin goes up, the subject reaches out and begins turning the tap down to reduce the hot water flowing into the shower. At a certain point the perfect adjustment is reached but the subject does not realize that until the water with the correct temperature has had time to go up the pipe and out the showerhead to the temperature receptors. During this delay, the subject continues to turn the flow of hot water down until the temperature of the water coming out of the showerhead is much too cold. When that cold water reaches the skin the subject reaches out again and winds the tap the other way, but once again overshoots because of the delay in the pipe and allows the temperature to get too hot. It is clear that a system like this could continue to oscillate indefinitely. It is also intuitively clear that oscillations will be more likely to happen and will be bigger when they do occur if the subject has very sensitive skin, because in that case he or she will react much more briskly to small changes in temperature and wind the tap much more quickly round, causing a much bigger overshoot.

The main factors that will make this system unstable are therefore are the *delay* in the pipe and the sensitivity or the *gain* in the feedback loop, that is the amount of change in the flow of water that results from a certain change in temperature at the temperature receptor level.

The feedback control system for arterial P_{CO2} is analogous. Arterial P_{CO2} depends on the balance between flow of $CO_{2:}$ into the lung from venous blood and out of the lung, by means of ventilation. Arterial CO_2 is sensed by chemoreceptors. There is a delay between the change in ventilation and the change in CO_2 at the chemoreceptor. This feedback system can therefore become unstable if the gain in the CO_2 feedback loop is high or if the delay is increased.

This kind of system lends itself to mathematical modeling. Numerous researchers with background in engineering have developed mathematical simulations of the respiratory

feedback system. (2,4,5) Increasing sophistication of these models has meant that the various physiological mechanisms that are important to the controller have been described more and more accurately, with numerical constants based on better and better physiological data and an increasing number of adjustable components. The mathematical models become more complex as more variables are introduced, the difficulty of the mathematics increases, and major computational ability is required. An ideal model would accurately simulate all of the important components of the feedback system and would behave exactly like the physiological system it is intended to represent. All models so far have had their limitations. However, the behavior of even a moderately complex feedback system depends on interactions between the variables that are so complex that intuition proves to be a very unreliable guide to understanding how the system works. These models can therefore give us new ideas about mechanisms and how they work. In this paper, I will present some very interesting results from the most recent and most sophisticated model, developed by Zbignew Topor, John Remmers and associates. (5)

The model includes a lung, a central nervous component with brain and CSF, and a peripheral tissue component. These are linked by vascular loops, with each its own delay. The details included are a lung with variable volume, a dead space that varies with tidal volume, and blood with accurate representations of Bohr and Haldane effects, changes with the temperature and bicarbonate and interaction of all these things on oxygen carrying capacity. It has a chemosensor system with both oxygen and CO_2 sensitivity and their interaction. There are two chemoreceptors, one peripheral and one central, with different sensitivities and delays. Brain blood flow and metabolism are variable and depend on oxygen and CO_2. The mathematical model takes the form of multiple differential equations. In this model, any of the descriptive constants can be changed, for example carbon dioxide sensitivity, oxygen sensitivity, cardiac output, cerebral blood flow or inspired oxygen. (5)

One way of testing the model is to determine how it responds to a change in inspired oxygen. Figure 1 shows it behaves if, after five minutes at resting conditions, it is exposed to a sudden drop in expired oxygen, equivalent to having a subject ascend suddenly to high altitude. Arterial oxygen falls and ventilation rises. With this, arterial CO_2 drops rapidly and approaches a constant level of 25. After 50 minutes, the system begins to oscillate and the oscillations increase in amplitude. As shown in the expanded tracing of Figure 2, ventilation eventually oscillates between 20 liters a minute and zero, a pattern of recurrent central sleep apnea.

To test whether the model is stable under any circumstance, a method is to set all of the parameters, allow the system to run at stable ventilation and then perturb it, similar to flushing a toilet in the shower model. The perturbation used by Topor et al (6) was a brief drop in arterial CO_2 the equivalent of a single deep breath. When that is done ventilation immediately begins to oscillate. If the system is stable, the oscillations rapidly die away and steady breathing resumes. If it is unstable, the oscillations increase in size and a long-term pattern of periodic breathing is produced.

The introduction of two chemoreflex feedback loops with different gains and different delays has a surprising effect on behavior of the system. This is shown in Figure 3, a plot of central chemoreceptor sensitivity on the Y-axis and peripheral central chemoreceptor sensitivity on the X-axis. The sensitivity of 1.0 is normal. For this plot, all of the other parameters were set at their normal values. Inspired oxygen is normal for sea level. The model was then run many times with different combinations of central and peripheral

chemosensitivity. With each setting it was tested with a disturbance equivalent to a deep breath. The plot shows circumstances when it was stable or unstable. In the central part of the graph, indicated by dark triangles, the system was stable. In the top and right side of the graph, the system was unstable. The conclusion from examining this plot is that chemoreceptor gain at sea level can be increased to more than 3 times normal without making the system unstable if both central and peripheral receptors increase their gain together. This puts the system near the upward and rightward tip of the stable area. The system will oscillate however if central chemosensitivity is high while peripheral chemosensitivity is low, or vice versa.

An explanation for this unexpected interaction of chemo-sensor feedback loops comes from recognizing that the way the signals from the central receptor and peripheral receptor interact depends on their relative delays. The central nervous system hears about a drop of oxygen in arterial blood at the peripheral chemoreceptor much earlier than it hears about the same blood arriving at the central chemoreceptor. Each of these chemoreceptors in periodic breathing is sending out an oscillating signal. Both the signals have the same period (cycle length), but there is a phase difference. At a certain point in the breathing cycle, the central nervous system controller receives a signal from the peripheral chemoreceptor when oxygen partial pressure in the carotid artery is at its peak. At that moment, oxygen partial pressure at the central chemoreceptor may be still in a trough. The two signals are then giving opposite messages and to some extent will cancel each other out. How the signals add depends on both their relative gains and their relative delays. As a special example, if the gains in the two receptors are the same but one is delayed exactly one half of the breathing cycle behind the other, the oscillations in the two signals from chemoreceptors will completely cancel each other out at the central nervous system, giving the brain the impression that blood oxygen or CO_2 tensions are constant through the cycle.

It is of interest that the oscillations predicted by the model in a subject at sea level with high central chemoreceptor gain and low peripheral chemoreceptor gain (point B in Figure 4) have characteristics similar to the Cheyne-Stokes breathing in a period of nearly one minute. On the other hand, a system with a high peripheral chemoreceptor gain and a low central chemoreceptor gain has a much shorter period, around 20 seconds, which is more like altitude periodic breathing.

To apply this model to altitude breathing, it will be necessary to construct plots like Figure 3 for different altitudes by changing the fixed value of inspired oxygen to correspond to some altitude and then testing the system for stability at various combinations of chemoreceptor gains. It can be expected that the zone of stability will shrink with increasing altitude. The original example of central apnea provoked by hypoxia shown in Figure 2 indicates that a normal system should, if the model is correct, be unstable at the (1.0,1.0) point on this graph (normal sensitivity of both central and peripheral chemoreceptors) at high altitude. However, the exact shape of the zone of stability remains to be calculated different altitudes. Matching observations of breathing in sleep at various altitudes with such predictions of the model will be an interesting test of the validity of the model.

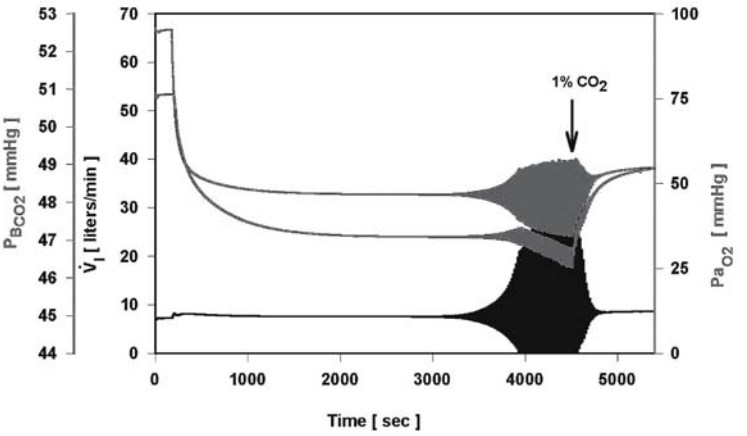

Figure 1. Response of ventilation and arterial oxygen and carbon dioxide to a sudden drop in inspired oxygen tension.

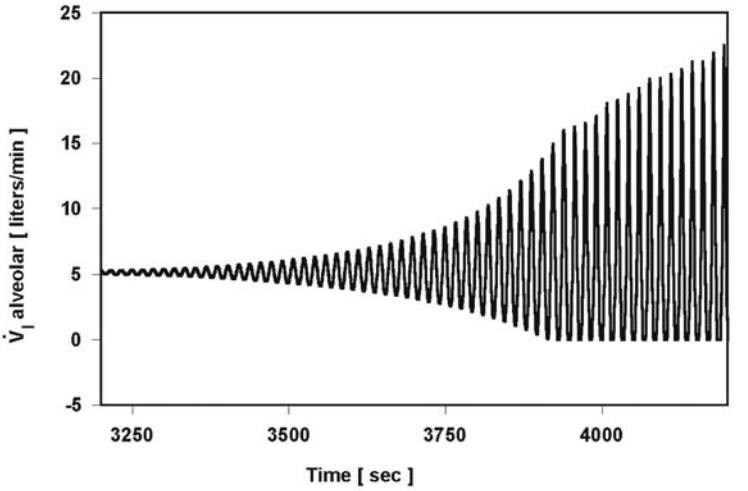

Figure 2. An expanded tracing of the ventilation tracing from Figure 1.

The model also gives hints about effect cerebral blood flow and varying cerebral blood flow on stability of breathing. An increase in cerebral blood flow does two things. It reduces the difference between PCO_2 and arterial blood and PCO_2 at the chemoreceptor itself, which is not in the blood stream but in adjacent tissue. In so doing, an increase in flow reduces the level of CO_2 stimulation at the chemoreceptor. Second, with higher flow, the wash-in and wash-out time for cerebral tissue PCO_2 is faster so that delay is effectively shorter. Cerebral blood flow is known to oscillate during periodic breathing.

When stability plots were done with cerebral blood flow twice normal, the area of stability was decreased. That is, the system was likely to oscillate at lower values of chemo-

receptor gains. On the other hand, when cerebral blood flow was reduced to half normal and its ability to react to oxygen and CO_2 was eliminated, (intended to simulate cerebral vascular disease) the system became more stable, with stability independent of peripheral chemoreceptor gain, (but still unstable at very high chemoreceptor gain.)

It is very important to emphasize that mathematical models are never complete or perfectly accurate. One cannot draw conclusions with any certainty. However, they do show that even relatively straightforward additions to the system such as two chemoreceptor loops can produce surprising behavior that would be hard to predict by intuition.

The model of Topor et al. lacks a number of elements that are thought to be of possible importance in control of breathing and in generation of periodic breathing. A partial list of missing elements is: 1. After-discharge or post stimulus potentiation. This is a tendency of the mammalian respiratory control system, after a sudden change in chemical stimulus, to continue breathing the way it had been breathing. For example, a sudden drop in CO_2 is followed by a relatively slow decline in ventilation to the new value. 2. As carbon dioxide is lowered, breathing eventually stops at the apnea threshold. When the CO_2 is increased again, however, apnea does not resume at the same threshold level but at a somewhat higher one. This hysteresis in the apneic threshold would intuitively be expected to make the system les stable (3). 3. There are other feedback loops, in particular mechanical feedback, in which simple expansion and contraction of the thorax with breathing has an inhibitory effect on the respiratory control centers. 4. The mechanics of the thoracic pump are not taken into account. In particular, the upper airway constitutes a resistance to breathing. It is a variable resistance depending on tone of upper airway dilator muscles. A controller that increases upper airway dilator tone at the same time that it increases diaphragm contraction will have a higher gain than a system that always has the same upper airway resistance. Investigating these possibilities with mathematical models may soon give us a clearer idea of the interaction of all the variables and possibly some new and unexpected concepts.

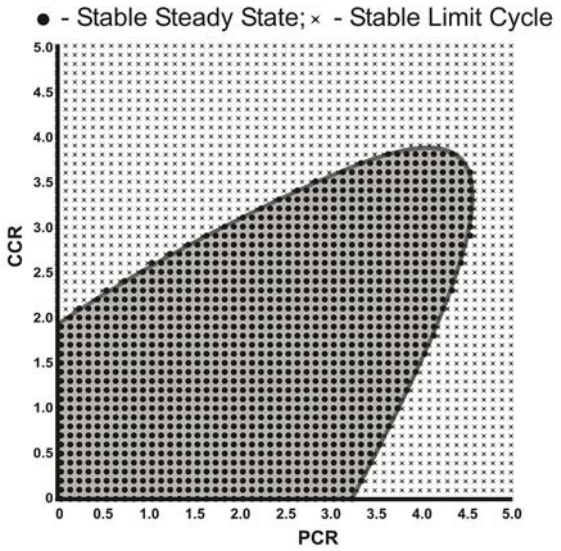

Figure 3. Conditions of central and peripheral chemoreceptor gain for which the model of the respiratory system is stable or unstable.

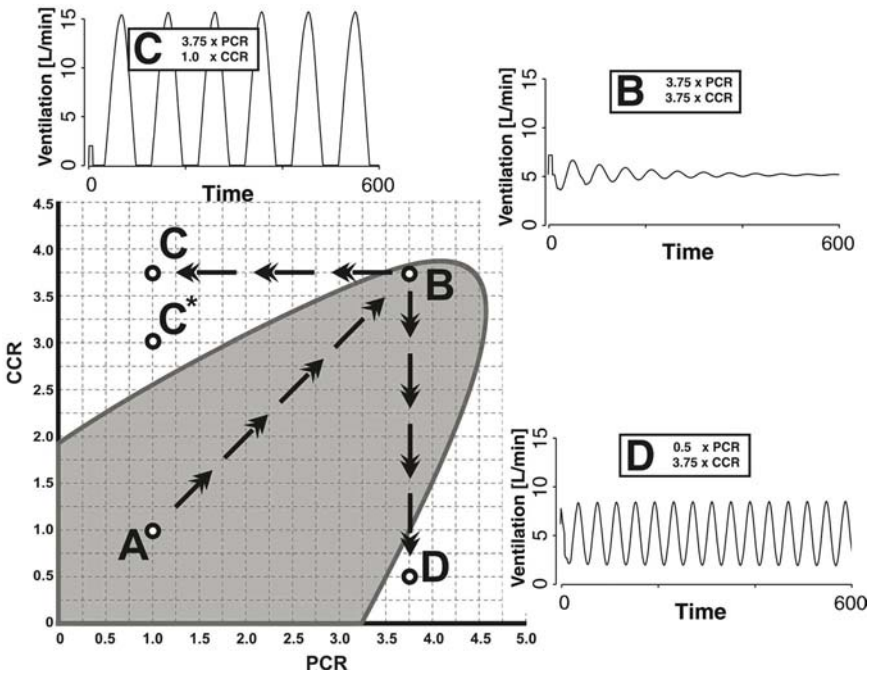

Figure 4. Same as Figure 3 showing examples of oscillations in ventilation for two points in the zone of instability.

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Chapter 7

CONTROL OF CEREBRAL BLOOD FLOW DURING SLEEP AND THE EFFECTS OF HYPOXIA

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Abstract: During wakefulness, cerebral blood flow (CBF) is closely coupled to regional cerebral metabolism; however CBF is also strongly modulated by breathing, increasing in response to both hypercapnia and hypoxia. During stage III/IV non-rapid eye (NREM) sleep, cerebral metabolism and CBF decrease whilst the partial pressure of arterial CO_2 increases due to a reduction in alveolar ventilation. The reduction in CBF during NREM sleep therefore occurs despite a relative state of hypercapnia. We have used transcranial Doppler ultrasound to determine middle cerebral artery velocity, as an index of CBF, and have determined that NREM sleep is associated with a reduction in the cerebrovascular response to hypercapnia. This reduction in reactivity would, at least in part, allow the observed reductions in CBF in this state. Similarly, we have observed that the CBF response to hypoxia is absent during stage III/IV NREM sleep. Nocturnal hypoxia and hypercapnia are major pathogenic factor associated with cardio-respiratory diseases. These marked changes in cerebrovascular control that occur during sleep suggest that the cerebral circulation may be particularly vulnerable to cardio-respiratory insults during this period.

Key Words: transcranial Doppler ultrasound, middle cerebral artery velocity, cerebral perfusion, hypoxia, hypercapnia

INTRODUCTION

Sleep is a reversible state of reduced responsiveness to the external environment. As sleep is widely observed within the animal kingdom, it is likely that the processes oc-

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curring during this state are of functional importance and have a significant survival advantages. In spite of this, the actual function of sleep remains speculative; many theories have been proposed including energy conservation (3), thermoregulation (25), neuronal plasticity (19), and memory processing (37). It is now widely accepted that the brain, and its cellular function, is most affected by the sleep process (24).

The brain produces energy by the metabolism of oxidisable substrates; however, as the brain contains no endogenous stores of energy, it is dependent upon cerebral blood flow (CBF) for constant replenishment of oxygen and glucose and the removal of waste. Therefore, CBF is tightly regulated to meet the brain's metabolic demands. In addition, CBF must respond to alterations in the blood gas status of the blood and must safeguard the brain from fluctuations in systemic arterial pressure. When cerebral perfusion falls below a level where homeostasis can compensate, cerebral ischemia and infarction may result (6). Sleep is a state in which disturbances in cardiovascular and respiratory regulation can be particularly evident, for example in sleep-disordered breathing. Such disturbances may particularly affect cerebral perfusion and it is therefore of importance to understand the regulation of CBF during sleep. The purpose of this review is to highlight findings from some of our recent studies to investigate CBF regulation in humans, in particular its regulation during non rapid eye movement (NREM) sleep.

CBF RESPONSE TO HYPERCAPNIA DURING SLEEP

A number of studies have determined CBF during stable stage III/IV, NREM sleep in healthy humans. The predominant observation is that CBF is reduced in this state compared with wakefulness (9, 13, 20, 23, 34, 40). This reduction is believed to be linked to a decrease in the metabolic demand of brain tissue in that state (23) however this link has not been directly tested.

Carbon dioxide is a potent cerebral vasodilator and, in humans during wakefulness, CBF is very sensitive to changes in arterial PCO_2, with cerebral vascular reactivity to hypercapnia ranging from about 3.5 to 5 % increase in CBF per mmHg increase in arterial CO_2, over the normal physiological range (11, 15, 29). With sleep, ventilation is reduced and arterial PCO_2 is increased. The reported reduction in CBF during stage III/IV, NREM sleep therefore occurs despite a relative state of hypercapnia (Fig 1). This reduction would not be predicted from the wakefulness effects of CO_2 and could at least in part, be explained by a reduction in the cerebral vascular reactivity to CO_2 during sleep. Our laboratory has investigated the effects of stable stage III/IV, NREM sleep on the CBF response to CO_2 using transcranial Doppler ultrasound to determine middle cerebral artery velocity as an index of CBF (26). We determined that, in normal human subjects, hypercapnic cerebral vascular reactivity is reduced by 70% compared to wakefulness (Fig 2). This marked reduction in cerebral vascular reactivity during sleep indicates that the regulation of CBF is significantly altered compared with wakefulness. The functional advantage of such a reduction in the sleep-related cerebral vascular reactivity is not immediately apparent and, as the cerebral vascular reactivity reflects the cerebral circulation's ability to adapt to the metabolic requests of the brain, any reduction could be perceived to increase the risk of cerebral ischemia and stroke.

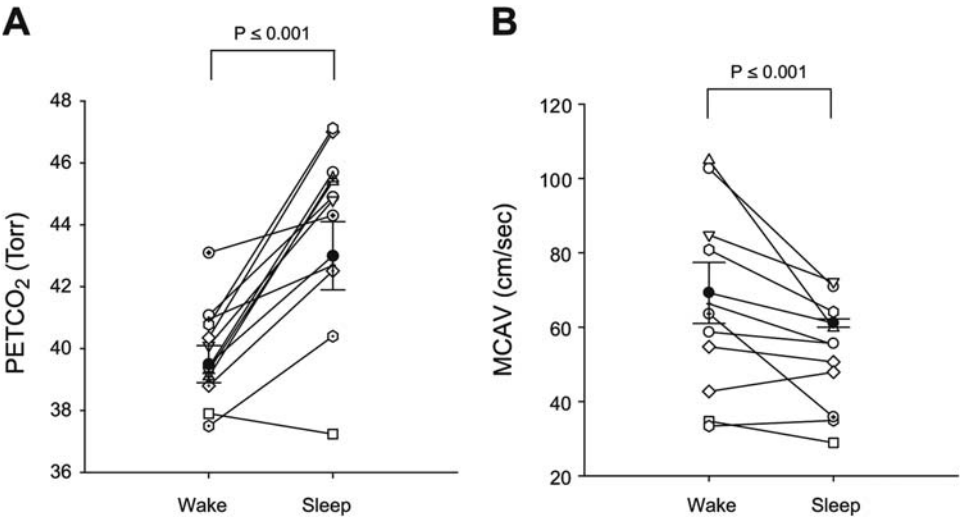

Figure 1. Sleep-related changes in PCO_2 and cerebral perfusion. (a) End tidal PCO_2 ($PETCO_2$) and (b) middle cerebral artery velocity (MCAV) during wake and stages III/IV NREM sleep. $PETCO_2$ was significantly greater, and MCAV significantly less, during sleep compared to wake. Open symbols: individual values; Solid symbols: group mean values (+/- sem). Reproduced, with permission, from (26).

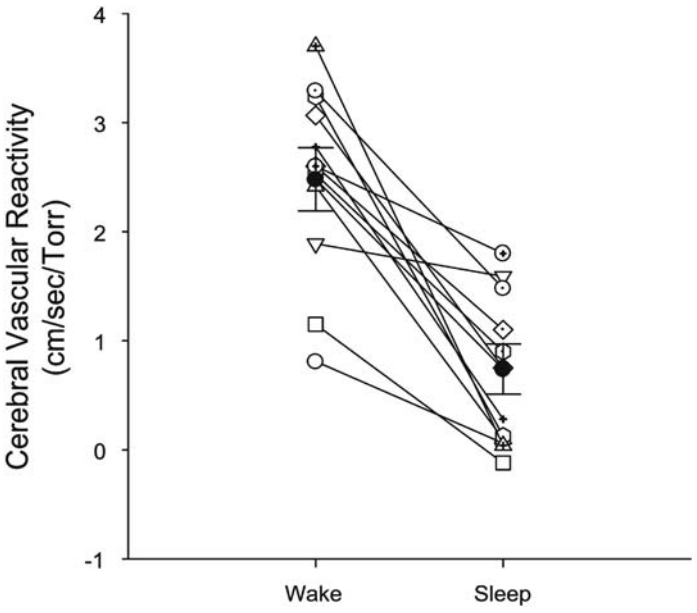

Figure 2. Changes in cerebral vascular reactivity from wake to sleep. The cerebral vascular reactivity to CO_2 from wake to NREM sleep is reduced in each individual. Open symbols: individual values; solid symbols: group mean values (+/- sem). Reproduced, with permission, from (26).

CBF RESPONSE TO HYPOXIA

Hypoxia has a well recognized vasodilator effect on the cerebral circulation (1, 7, 15). Although hypoxia is not present during wakefulness or sleep in healthy individuals, except at altitude, nocturnal hypoxia is a major pathological factor associated with cardiorespiratory diseases including obstructive sleep apnea (OSA) and congestive heart failure. Recently OSA, a condition in which cognitive function can be substantially impaired, has been associated with pathological loss of cortical grey matter (22, 28) suggesting that the nocturnal hypoxia associated with OSA may be sufficient to damage brain tissue directly.

During any hypoxic insult, protection of the brain will depend on an adequate cerebral vascular response. Normally, perfusion of the brain is dependent on a tight coupling between its oxygen supply and the metabolic demand (38). During wakefulness, a decrease in oxygen supply results in a decrease in cerebral vascular tone and a consequent increase in CBF that will mitigate the effects of the systemic hypoxia. We recently investigated the effects of isocapnic hypoxia on CBF (27). As expected CBF increased in response to hypoxia during wakefulness; in contrast, during NREM sleep, the CBF response to hypoxia was absent (Fig 3). This data is further evidence that the regulation of CBF during sleep is significantly altered compared with the waking state. As the brain is particularly sensitive to the effects of hypoxia, the inability of the cerebral vasculature to respond to hypoxic stress during sleep suggests a significant vulnerability of the brain in this state.

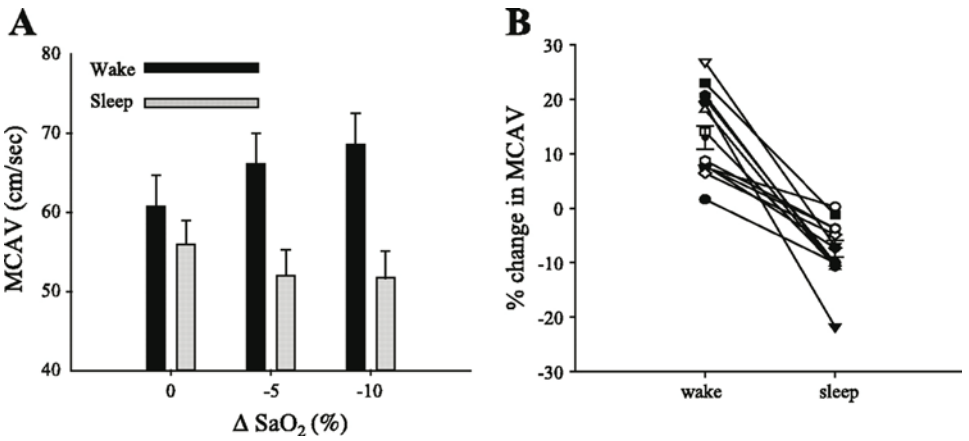

Figure 3. Reductions in cerebral vascular response to hypoxia during Stage III/IV NREM sleep. (a) Middle Cerebral Artery Velocity (MCAV) during baseline isocapnic euoxia, -5% and -10% SaO_2 (isocapnic hypoxia) during wakefulness (black bars) and sleep (grey bars; mean ± sem, n = 13). (b) Percentage changes (from baseline isocapnic euoxia to isocapnic hypoxia; -10% SaO_2) in MCAV (cm/sec) during wakefulness and sleep for each individual and for the group mean (filled circle with sem bars). Reproduced, with permission, from (27).

POTENTIAL MECHANISMS UNDERLYING CHANGES IN CEREBROVASCULAR REACTIVITY DURING SLEEP.

The mechanisms underlying the CBF response to hypercapnia and hypoxia during wakefulness remain to be elucidated; several vasoactive endothelial mediators have been proposed including prostanoids, nitric oxide, adenosine, and ATP sensitive potassium channels (4, 12, 30, 33, 35, 42, 43). Whether sleep exerts a direct effect on the production and activity of these mediators is unknown. Evidence does exist to suggest that nitric oxide is under circadian regulation, with changes during sleep that are consistent with our observed reductions in cerebrovascular reactivity (10).

During wakefulness and under physiological conditions the involvement of the central nervous system in the regulation of cerebral circulation, is controversial (14, 21). Despite this, the onset of sleep is associated with an overall reduction in neural activity that might modulate cerebral perfusion. In addition, central catecholaminergic and cholinergic systems located within the brainstem have been shown to modulate changes in cerebral vascular reactivity to CO_2. For example, cerebral vascular reactivity to CO_2 is reported to be lower following the destruction of the locus ceruleus and the ascending reticular activating system (2, 39). As these brainstem areas are intimately involved in sleep/wake functions, it is possible that changes in sleep state acting via these areas could modulate the changes in cerebral vascular reactivity.

CBF AT SLEEP ONSET

Less consistent changes in CBF have been reported during sleep onset and light sleep, than during stage III/IV sleep. One study reported that CBF was maintained at the same level during stage I sleep as wakefulness and then decreased progressively from stage II to IV NREM sleep (13). Others have reported transient increases in CBF during stage II sleep (20). A study using positron emission tomography determined regional CBF changes during stage I sleep and demonstrated relative flow increases in the occipital lobes (17). These observations suggest that the regulation of CBF during sleep onset may differ from that of established sleep; however none of these studies related the changes in CBF to the rapid changes in cortical activity that occur during this period.

Sleep onset can be considered to begin with the first occurrence of slowing of the EEG, the appearance of relatively low voltage mixed frequency EEG containing a predominance of theta activity and the absence of rapid eye movements. Sleep onset ends at the beginning of stable stage II sleep (32). During this phase the EEG contains spontaneous fluctuations in alpha and theta rhythms, including alpha-theta transitions, and theta-alpha transitions, which can be considered as transitions from wakefulness to sleep and spontaneous awakening respectively (5, 8, 41). These fluctuations in cortical state that occur during this phase are associated with marked changes in cardio-respiratory control. In particular, ventilation decreases with the alpha-theta transition and increases with the theta-alpha transition (5, 8, 41). With this context, we hypothesized that changes in blood flow would be related to changes in cortical activity, specifically, that CBF would decrease at the alpha-theta transition and increase at the theta-alpha transition (18). Contrary to our expectation, CBF

increased with the alpha-theta transition (transition to sleep; Fig. 4a), coincident with a reduction in ventilation and blood pressure. Conversely with the theta-alpha transition (spontaneous awakening) CBF decreased (Fig 4b), coincident with an increase in ventilation and blood pressure, the change in CBF being more abrupt than the change with the alpha-theta transition. The increase in CBF following the alpha-theta transition might be secondary to the increase in $P_{ET}CO_2$ associated with the reduction in ventilation. However, our observations are not consistent with this interpretation. First, the CBF increase was rapid compared with the slow rise in $P_{ET}CO_2$. In addition, the relative changes in MCAV to $P_{ET}CO_2$ at the alpha-theta transition is much higher that that predicted by the hypercapnic cerebral vascular reactivity during either wakefulness or stage III-IV NREM sleep (26). Similarly the increase in MCAV following the theta-alpha transition cannot be attributed to the fall in $P_{ET}CO_2$.

The increase in CBF following the alpha-theta transition occurred despite a fall in arterial blood pressure and the decrease in CBF following the theta-alpha transition occurred despite a rise in blood pressure. Thus, it appears that the alpha-theta transition is directly associated with cerebral vasodilatation and the theta-alpha transition with cerebral vasoconstriction. As these changes in cerebral vascular tone cannot be due to the indirect effect of cardiovascular or respiratory factors, they may be directly related to changes in cortical state reflected in the changes in alpha and theta rhythms. The mechanism for such fluctuations in cerebral vascular tone during sleep onset period is not known. However, these observations suggest that the changes in CBF during sleep onset are regulated by different mechanisms from those that regulate long-term reduction in CBF associated with stable NREM sleep.

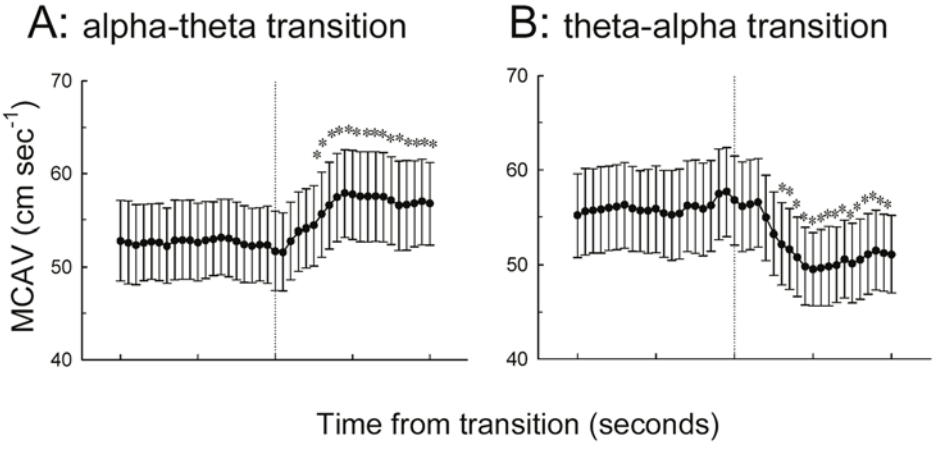

Figure 4. Middle cerebral artery velocity (MCAV) changes during sleep-wake transition; A: increase in MCAV following the alpha-theta transition, B: decrease in MCAV following the theta-alpha transition (mean ± sem; n=10; *p<0.01 compared with the pre-transition baseline. Data from (18).

CLINICAL IMPLICATIONS

Nocturnal hypoxia and hypercapnia are characteristics of cardio-respiratory diseases

such as OSA and congestive heart failure. So far, our laboratory has demonstrated that cerebral vascular responses to hypercapnia and isocapnic hypoxia are drastically reduced or even abolished in healthy subjects during NREM sleep. Failure of the cerebral circulation to respond to hypoxia and hypercapnia would result in hypo-perfusion of the brain leading to impaired neural function and increased risk of cerebral ischemia and stroke (31), a condition with an increased frequency during the early hours of the morning. Such sleep related reductions in cerebral vascular reactivity may be partly responsible for the pathological loss of grey matter and cognitive dysfunction reported within OSA (22, 28) and congestive heart failure (44).

During sleep onset, the spontaneous fluctuations in alpha and theta rhythm are related to instability in breathing in normal subjects (16, 36) as well as patients with sleep disordered breathing. Whilst the increase in CBF may be interpreted as a benefit, to maintain an adequate perfusion against acute hypoxia, hypercapnia and systemic hypotension at the transition from wake to sleep, the increase in CBF may cause a reduction in extracellular carbon dioxide concentration in the brain, resulting in a decrease in central chemoreceptor stimulation, which may promote breathing instability and central apnoea. Conversely, the reduction in CBF during a spontaneous arousal during the sleep onset period, may increase chemo-stimulation, and produce an excessive increase in ventilation and breathing instability.

CONCLUSION

In conclusion, our studies indicate that the compensatory increases in CBF, in response to hypercapnia and hypoxia, are reduced or abolished in healthy humans during stable stage III/IV, NREM sleep compared to wakefulness. These findings suggest a major state-dependent vulnerability associated with the control of the cerebral circulation. The inability of the cerebral circulation to respond to the metabolic demands of the brain could therefore be linked to an increased risk of cerebral ischemia and stroke during this time.

The marked changes in CBF during the period of sleep onset could, in part, explain the relative breathing instability reported during this period. In addition, assuming that the cardio-respiratory response at the theta-alpha transition during sleep onset is similar to the arousal from stage II, slow-wave (stages III/IV) or REM sleep, these reductions in CBF with arousal would indicate a significant inability of the cerebral vascular circulation to respond to the cardio-respiratory changes induced by conditions such as sleep-disordered breathing.

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Chapter 8

NEURAL CONSEQUENCES OF SLEEP DISORDERED BREATHING: THE ROLE OF INTERMITTENT HYPOXIA

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Abstract: Sleep disordered breathing is characterised by periodic breathing, episodes of hypoxia and repeated arousals from sleep; symptoms include excessive daytime sleepiness, impairment of memory, learning and attention. Recent evidence from animal studies suggests that both intermittent hypoxia and sleep fragmentation can independently lead to neuronal defects in the hippocampus and pre frontal cortex; areas known to be closely associated with neural processing of memory and executive function. We have previously shown that sleep disordered breathing is associated with loss of gray matter concentration within the left hippocampus (47). We have now confirmed and extended this finding in 22 right handed, newly diagnosed male patients (mean (sd): age 51.8 (15.4) yrs, apnea / hypopnea index 53.1 (14.0) events/hr, minimum nocturnal oxygen saturation 75 (8.4) %) and 17 controls matched for age and handedness. Voxel-based morphometry, an automated unbiased technique, was used to characterise changes in gray matter concentration. The magnetic resonance images were segmented and grey matter concentration determined voxel by voxel. Analysis of variance was then preformed, adjusted for overall image intensity, with age as a covariant. Additional to the deficit in the left hippocampus, we found more extensive loss of gray matter bilaterally in the parahippocampus. No additional focal lesions were seen in other brain regions. Based on our findings and data from other human and animal studies, we speculate that in patients with sleep disordered breathing intermittent hypoxia is associated with neural deficit, and further that such lesions may lead to cognitive dysfunction.

Key Words: sleep apnea, periodic breathing, cognitive, memory

Hypoxia and Exercise, edited by R.C. Roach *et al.*
Springer, New York, 2006.

INTRODUCTION

Sleep disordered breathing may present in several forms (I) obstructive sleep apnea / hypopnea, resulting from occlusion or partial closure of the pharyngeal airway (II) central sleep apnea, resulting from reduced efferent output to the respiratory pump muscles (III) mixed apnea, a combination of both obstructive and central events (IV) Cheyne-Stokes respiration: a periodic breathing pattern that is characterised by a crescendo-decrescendo fluctuation in tidal volume and may contain a central apnea or hypopnea. Obstructive sleep apnea / hypopnea is the most common form of sleep disordered breathing occurring in 1-4% of the middle-aged western male population (61, 67), increasing up to 24-30% in elderly males (37). Central sleep apnea and Cheyne-Stokes respiration are more often seen in patients with heart failure, or during conditions of chronic hypoxia as occurs at altitude. All forms of apnea and hypopnea produce intermittent hypoxia, to a greater or lesser extent (Figure 1).

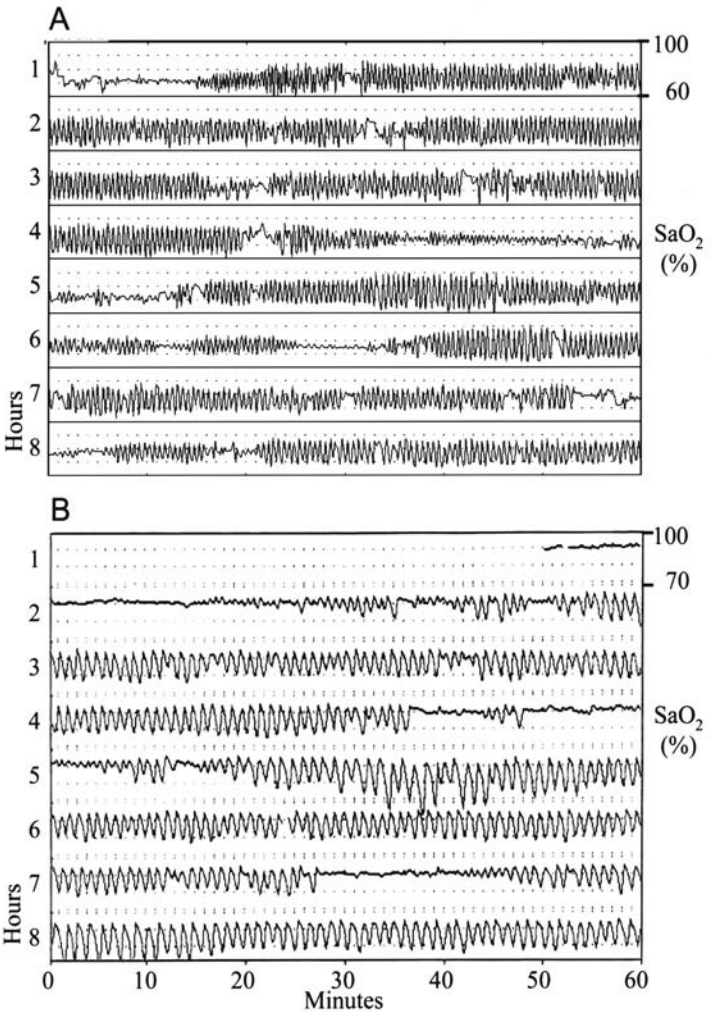

Figure 1. Intermittent hypoxia associated with obstructive sleep apnoea (Bottom Trace) and chronic hypoxia of altitude (Top Trace).

Apneic and hypopneic events are usually terminated by an arousal from sleep. As a result, sleep disordered breathing is typically associated with poor nocturnal sleep quality and excessive daytime sleepiness. The term obstructive sleep apnea / hypopnea syndrome (OSAHS) is usually taken to mean patients presenting with an apnea / hypopnea index (AHI) > 5 events / hour together with excessive daytime somnolence, not explained by other factors, or any two of the following factors: choking or gasping during sleep, recurrent awakenings during sleep, unrefreshing sleep, daytime fatigue, impaired concentration.

COGNITIVE FUNCTION AND SLEEP DISORDERED BREATHING

Cognitive dysfunction includes the impaired ability to problem solve, manipulate information, plan, inhibit responses and maintain attention (14, 15). It is a frequently reported problem in patients with sleep disordered breathing, with over 50% having some form of memory impairment (34). The mechanisms leading to the cognitive and memory deficits are unclear. Excessive daytime somnolence may result in an inability to focus and sustain attention (44), put simply patients fall asleep during boring repetitive tasks. Alternatively, sleep deprivation may lead to impaired memory by altering neural function (31). Cognitive dysfunction may be a consequence of hypoxia related neuronal deficits, in particular intermittent hypoxia (56). These mechanisms will be addressed fully in the latter sections of this paper. In the remainder of this section we will review in more detail the relationship between sleep disordered breathing and aspects of cognitive function.

Previous research has examined many aspects of cognitive functioning in patients with sleep disordered breathing, including facets of memory, attention, vigilance and executive functions. In patients with mild sleep disordered breathing (AHI 10-30 events/hour) some, but not all aspects of working memory (e.g. the ability to hold information long enough to make use of it, such as a phone number) are deficient compared to healthy controls (AHI < 5 events / hour) (54). In patients with more severe OSAHS, the recall of word lists is reduced and these patients make less efficient use of semantic cues to assist their memory. However, recognition memory and retention of previously learned information are not significantly affected (58). Taken together, these data suggest that not all aspects of memory are equally affected by sleep disordered breathing, and that the severity of the disease may influence the extent of the dysfunction.

In an important study into the effects of OSAHS on pre frontal lobe-related executive functions, Feuerstein et al found that patients were significantly impaired on a number of functions; in particular, deficits where found in tests of planning and flexibility, plus tests requiring inhibition of a learned response (21). In addition, phonemic fluency, another frontal lobe task, is impaired in OSAHS patients, compared to controls matched for age and educational status (58).

If sleep disordered breathing is associated with impairment of cognitive tasks involving the pre frontal cortex, it is reasonable to question: *to what extent treatment reverses the cognitive dysfunction?* The treatment of choice for OSAHS is continuous positive airway pressure (CPAP). This treatment has been successfully used to reduce the number of apneas / hypopneas, and the number of respiratory-related arousals, which in turn improves the quality of sleep. Patients who use CPAP frequently report a reduction in daytime som-

nolence, which is confirmed objectively with an increase in sleep latency (59). However, a summary review of the effectiveness of CPAP in improving cognitive function is confounded by the fact that different researchers have used different cognitive test batteries, and CPAP treatment for varying periods of time; additionally, compliance is frequently poor or not reported. Nevertheless, OSAHS patients using CPAP treatment have shown an improvement in some aspects of cognitive function. In particular, mental flexibility, such as trail making B (17, 18), psychomotor vigilance (17, 35) and measures of attention (20, 21) all improve following CPAP treatment.

The duration of CPAP treatment required to reverse cognitive dysfunction varies. Significant improvements in tests of sustained attention, spatial memory, motor agility and dexterity have been shown after fifteen days of CPAP treatment (20). When examined again at four months, these improvements had been maintained but no further significant improvements had been made. Some cognitive deficits noted prior to CPAP were not reversed by treatment; these included tests of executive function. These data are in accordance with other studies which have reported significant improvements in tests of vigilance after one night of CPAP treatment, with further significant improvements after fourteen nights (36) and six months of treatment (5); the latter study having no interim analysis at fourteen / fifteen nights.

Finally, it is important to note that not all studies of CPAP treatment have shown improvements in cognitive function. A placebo controlled study, examining the effects of CPAP on cognitive functioning after one week found no improvements in any neuropsychological tests used (2). The authors suggest that this may be due to the short duration of treatment use prior to evaluation, or poor compliance. However, this is unlikely since an earlier placebo controlled study of CPAP in OSAHS patients found that four weeks of treatment significantly improved objective and subjective measures of sleepiness, but failed to detect any improvement on the neuropsychological function (19). Similarly, sleepiness and cognitive function were compared in patients who did, and did not comply well with CPAP; in those who were compliant there was a significant improvement in sleepiness but not cognitive function (16). The results of these studies, compared to those mentioned earlier may be explained in part by the differences in cognitive test batteries. Nevertheless, the latter studies are placebo controlled and as such must be taken into account.

From this review of the literature we conclude that the use of CPAP in OSAHS leads to reversal of day time somnolence and vigilance; improvements in some, but not all aspects of cognitive dysfunction are seen in the majority of studies. We speculate that treatment of sleep disordered breathing may improve vigilance and aspects of memory by reversing daytime somnolence; whereas it has a reduced and inconsistent effect on the cognitive dysfunction, because cognitive function results from neurodegeneration associated with chronic intermittent hypoxia. This suggestion is supported by correlation studies which have shown that tests of executive functions and visuo-constructional ability correlate strongly with measures of nocturnal hypoxemia (6, 46). However, deficits in memory and attention correlated more strongly with measures of daytime somnolence (46).

STRUCTURAL CHANGES IN BRAIN MORPHOLOGY ASSOCIATED WITH SLEEP DISORDERED BREATHING

It is widely accepted that episodes of anoxia and / or hypoxia in humans can result in defuse neurodegeneration (33) and in selected cases, bilateral focal lesions of the hippocampus can occur (22). Conversely the effects of chronic intermittent hypoxia are less clear. In rats, exposure to intermittent hypoxia during sleep results in cellular damage within the CA1 region of the hippocampus and adjacent cortex (27, 55).

In humans several recent studies have investigated the suggestion that the intermittent hypoxia associated with OSAHS leads to changes in brain morphology (22, 39, 47, 48). In the first of these studies, Macey et al (39) reported widespread changes in gray matter concentration across the brain in twenty one patients with OSAHS compared to twenty one age matched controls; including the frontal and parietal cortex, the temporal lobe, anterior cingulate, hippocampus and cerebellum (Figure 2, top panel). In these patients, the decrease in gray matter concentration was related to the severity of the OSAHS disease. For each group the magnetic resonance images were analysed using voxel-based morphometry (VBM; Figure 2, top panel: A). This is an automated and unbiased technique that can be used to characterise changes in grey matter concentrations between groups (23, 26, 43, 65). It has the potential advantage of being able to detect subtle changes in relatively small structures such as the hippocampus (40). However, interpretation of the data obtained using this technique may be confounded by the statistical analysis. Macey et al (39) used a relatively low level of significance to report their findings; $p<0.001$, uncorrected for multiple comparisons, which may explain the wide spread gray matter loss in their study.

In the recent study of O'Donoghue et al (48) changes in brain morphology were examined in twenty five patients with severe OSAHS compared to twenty three healthy controls. Using VBM these authors found some reduction in gray matter concentration when the data were reported at $p<0.001$, uncorrected for multiple comparisons (Figure 2, bottom panel). However, when the data were analysed at $p<0.05$, corrected for multiple comparisons no significant reductions in gray matter in were seen. The authors corroborated their findings using a second method of MRI analysis; region of interest analysis (Figure 2, bottom panel: B)

The differences between the studies of Macey et al (39) and O'Donoghue et al (48) may in part be explained by differences in patient selection. The patients studied by O'Donoghue et al (48) had more severe OSA compared to those of Macey et al (39). However, this would have maximised the chances of O'Donoghue et al (48) finding changes in gray matter concentration. On the other hand, the study by Macey et al (39) included some patients known to have neurological and cardiovascular co-morbidity, most commonly hypertension; this could have had an independent effect on brain morphology and resulted in a reduction in gray matter concentration.

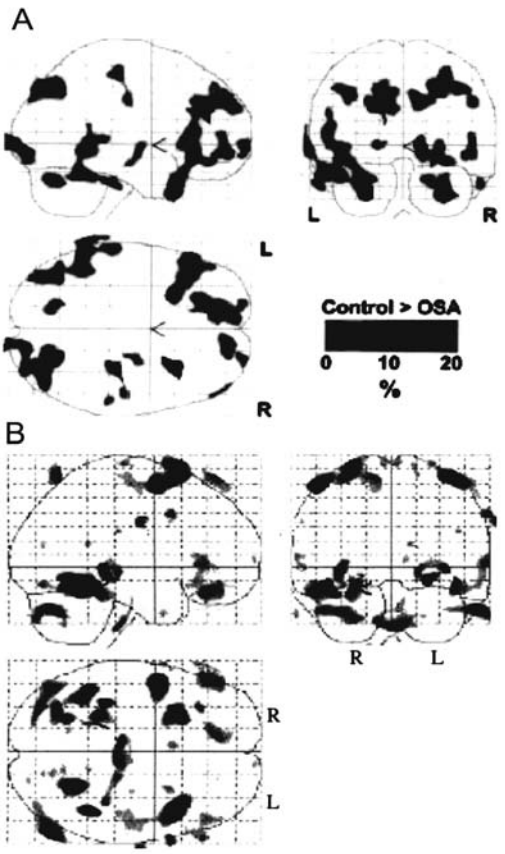

Figure 2. Glass brain display of regions of significantly reduced gray matter ($p<0.001$, uncorrected for multiple comparisons) in 21 subjects with OSAHS, weighted by disease severity and 21 healthy controls, matched for handedness and age (Macey et al (39) Top Panel and O'Donoghue et al (48).

Our group have also carried out a study to investigate the hypothesis that OSAHS is associated with changes in brain morphology, particularly a focal loss of grey matter within the hippocampus. Seven male right-handed, newly-diagnosed OSAHS patients were investigated using VBM. Compared to controls our study revealed a loss of grey matter concentration within the left hippocampus in patients with OSAHS (47). The level of significance used was $p=0.01$, corrected for multiple comparisons based on a hippocampal region of interest; this was selected on the basis of an *a priori* hypothesis of hippocampal gray matter loss in the OSA patients. We have now confirmed and extended this finding in twenty-two OSAHS patients compared to seventeen controls. See Table 1 for subject details. Additional to the deficit in the left hippocampus, this larger group of patients had more extensive loss of grey matter bilaterally in the para-hippocampus ($p<0.001$, a priori region of interest, uncorrected for multiple comparisons, with age as a covariant); no additional focal grey matter reductions were seen in other brain regions ($p>0.05$, corrected for multiple comparisons) (Figure 3). Five of our OSAHS patients and one of the control subject had a clinically diagnosed hypertension or were on antihypertensive medications. Therefore we carried out a second analysis removing these subjects. The focal lesions in the hippocampus and para hippocampus remained present on re-analysis.

Table 1. Subject characteristics.

	OSA patients (n=22)	Controls (n=17)
Age (years)	51.8 (15.4)	53.1 (14.0)
BMI (Kg/m^2)	32.4 (5.6)	24.8 (4.3)
Epworth Sleepiness Score	13.3 (4.2)	3.4 (1.2)
AHI (events/hour)	53.1 (14.0)	< 10
Minimum nocturnal oxygen saturation (%)	75 (8.4)	n/a

As we have alluded to above, sensitivity of VBM has been questioned by some. Therefore it is of significant interest that a fourth study has been published in which neuronal deficit in the hippocampus has been examined using another method of MRI analysis. In this study Gale and Hopkins (22) found bilateral hippocampal volumetric loss in 36% of the severe OSAHS patients they studied (n=14; respiratory disturbance index 84 (18) events / hour; mean percentage sleep time < 90% saturation 65 (34)%). The MRI scans were analysed using an alternative quantitative (volumetric) method (7).

So far we have discussed the impact of sleep disordered breathing on changes in gray matter concentration in humans. We conclude that some, but not all available data, supports the concept that OSAHS is associated with reduced gray matter in the hippocampus. However, before concluding this section we must mention that the relationship between OSAHS and with white matter disease has also been investigated (11, 13, 63). These studies have found no relationship between OSAHS and sub-clinical white matter disease (11, 63) or AHI and brainstem white matter disease (13).

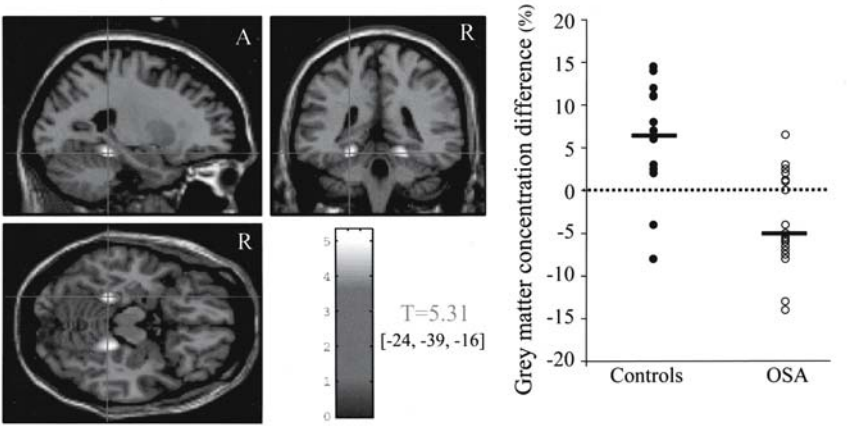

Figure 3. Shading indicates a statistically significant bilateral reduction in grey matter concentration within the para-hippocampus for the group of 22 OSAHS patients minus the 17 controls, overlaid on a standard brain template. Cross hairs indicate voxel of maximum significance ($p < 0.001$, *a priori* region of interest, uncorrected for multiple comparisons) at –24 (left), -16 (posterior), -34 (superior) mm, relative to the midline of the anterior commissure; no reductions were seen in other brain regions ($p>0.05$, corrected for multiple comparisons). Images oriented to a horizontal plane through the anterior and posterior commissures.

FUNCTIONAL IMPLICATIONS OF NEURONAL DEGENERATION IN THE HIPPOCAMPUS AND FONTAL CORTEX

To examine the functional implications of neuronal deficit in the hippocampus, studies have been carried out in patients with bilateral hippocampal degeneration, resulting from diseases other than sleep disordered breathing. In these patients aspects of memory are impaired. In one study, there was a reduction in recall of factual knowledge for several years prior to the onset of memory loss, although factual information acquired from greater than eleven years prior to onset of memory impairment remained intact (41). However, patients with bilateral hippocampal lesions do not always have complete loss of memory recall, suggesting either that other brain regions are recruited in the absence of hippocampal function, or that some cells in the hippocampus are spared (42).

Patients with damage limited to the hippocampus appear to show a reduction in source memory (41); the recognition of something as familiar and knowing the context in which it was first encountered (60). However, it must be noted that source memory is supported by other areas of the brain. Indeed studies have shown deficits in this function in patients with damage to the left prefrontal cortex, implicating this area as a vital component in the capacity for memory for source (24, 60).

Interestingly, the hippocampus has not been specifically implicated in working memory (10), whereas it does appear to be involved in spatial memory, particularly for navigation (40, 49). Bilateral dorsal hippocampal lesions have resulted in impaired spatial memory in rats, even when the lesion size encompassed as little as 30% of the total hippocampal volume (8). In addition, the medial prefrontal cortex has also been implicated in spatial memory, with lesions to either the hippocampus or the medial prefrontal cortex resulting in disruption of spatial memory capacity (62).

MECHANISMS OF NEURONAL DEGENERATION IN SLEEP DISORDERED BREATHING: THE ROLE OF INTERMITTENT HYPOXIA

As mentioned earlier at least two studies (6, 46), have examined the correlation between respiratory measures of disease severity, and performance on particular tests of cognitive function in order to address the question: *to what extent are cognitive deficits seen in patients with sleep disordered breathing attributable to hypoxemia, versus daytime somnolence?* In a large population based study, Adams et al (2001) have used factor analysis to show that respiratory disturbance index and nocturnal hypoxemia are significant predictors of declarative memory and signal discrimination (1). Furthermore, performances on tests which tap working memory, such as reverse digit span, were found to be predicted by the respiratory disturbance index (1). Performance on tasks involving attention, were significantly predicted by sleepiness (1). These data indicate that hypoxemia contributes to cognitive dysfunction in OSAHS. Consistent with this suggestion correlations between AHI and measures of visuo-spatial organisation (9, 29), visuo-motor coordination (9), reaction time (9), motor speed (29) and distractibility (9) have been shown.

From our study (47), we speculate that the changes in brain morphology which occur in patients with OSAHS resulted from the intermittent hypoxic insult. The mechanisms contributing to the hypoxic-induced hippocampal neurodegeneration are likely to have included several processes, with much of the recent research focused on the role of oxidative stress (3, 52, 53).

Brain function is critically dependent on oxygen supply. During any hypoxic insult, protection of the brain is dependent on a rapid cerebral vascular response otherwise cerebral ischemia with neuronal deficit will ensue. Recent research in healthy humans suggests that the cerebral vascular responses to both hypoxia and hypercapnia are significantly altered during stable non rapid eye movement sleep compared to wakefulness *(for a full review - see Corfield and Meadows Chapter 7 in this volume).*

Cellular responses to hypoxia include changes in the mediation ions channels, release of vasoactive substances from the endothelium and neighbouring tissues, regulation of gene expression and tissue remodelling (38). Hypoxia causes modulation of ion channels and in the hippocampal neurones immediate hyperpolerisation occurs via activation of the K_{ca} channels (32). Exposure to intermittent hypoxia during sleep results in cellular damage within the cerebella cortex (50), wake-promoting regions of the brainstem and basal forebrain (64), and CA1 region of the hippocampus and adjacent cortex (27, 30, 55). In the animals with hippocampal damage evidence of increased membrane lipid peroxidation and oxidative stress occurs, which is attenuated with administration of antioxidants (56). The susceptibility of the hippocampal neurones to neural dysfunction may be age dependent, with very young and elderly animals being more vulnerable (12, 28). The importance of this finding is that the prevalence of sleep disordered breathing increases with age (37) and in the young, sleep disordered breathing has been linked with attention deficit / hyperactivity disorder (4).

In animal experiments mentioned above the onset of intermittent hypoxia is abrupt, this is not the case in humans where the development of sleep disordered breathing is insidious. This is of interest since the neural degeneration in these experiments may be time-course dependent. In one study the neuronal deficit peaked at 3 days, returning to normoxic levels within 14 days in animals exposed to intermittent hypoxia (25). These findings lead to the suggestion that long-term potentiation, may play an important role in the impact of intermittent hypoxia on neural function (51).

MECHANISMS OF NEURONAL DEGENERATION IN SLEEP DISORDERED BREATHING: THE ROLE OF SLEEP DEPRIVATION

Although the focus of this paper is the role of intermittent hypoxia on the neural degeneration associated with sleep disordered breathing any review of sleep disordered breathing would be incomplete without considering the role of sleep *per se*.

Recent research has demonstrated the vulnerability of the hippocampus to damage in sleep deprivation (57). Selective REM sleep deprivation can also lead to a reduction in neuronal excitability in the CA1 pyramidal neurones, but not the dentate gyrus granule cells of the hippocampus (45). Sleep deprivation using a treadmill paradigm has also shown a

reduction in the proliferation of cells in the dentate gyrus (31). If sleep deprivation in and of itself can result in neuronal deficits it is interesting to speculate on the combined effects of sleep deprivation and intermittent hypoxia on neuronal plasticity in humans, an area which thus far has not been investigated.

CLINICAL IMPLICATIONS OF CHANGES IN MORPHOLOGY ASSOCIATED WITH OSAHS

As outlined earlier treatment of OSAHS using CPAP produces a considerable improvement in daytime somnolence (19). However, despite the obvious benefits of CPAP treatment in patients with severe OSAHS, there remains considerable debate as to the clinical and economic benefits of treating the large number of patients with relatively-mild disease (66). In concluding this paper we suggest that if sleep disordered breathing is associated with focal changes in brain morphology, it is possible that early treatment of the disease with CPAP may prevent structural changes and subsequent cognitive dysfunction in some patients.

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Chapter 9

FINDING THE GENES UNDERLYING ADAPTATION TO HYPOXIA USING GENOMIC SCANS FOR GENETIC ADAPTATION AND ADMIXTURE MAPPING

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Abstract: The complete sequencing the human genome and recent analytical advances have provided the opportunity to perform genome-wide studies of human variation. There is substantial potential for such population-genomic approaches to assist efforts to uncover the historical and demographic histories of human populations. Additionally, these genome-wide datasets allow for investigations of variability among genomic regions. Although all genomic regions in a population have experienced the same demographic events, they have not been affected by these events in precisely the same way. Much of the variability among genomic regions is simply the result of genetic drift (*i.e.,* gene frequency changes resulting from the effects of small breeding-population size), but some is also the result of genetic adaptation, which will only affect the gene under selection and nearby regions. We have used a new DNA typing assay that allows for the genotyping of thousands of SNPs on hundreds of samples to identify regions most likely to have been affected by genetic adaptation. Populations that have inhabited different niches (*e.g.,* high-altitude regions) can be used to identify genes underlying the physiological differences. We have used two methods (admixture mapping and genome scans for genetic adaptation) founded on the population-genomic paradigms to search for genes underlying population differences in response to chronic hypoxia. There is great promise that together these methods will facilitate the discovery of genes influencing hypoxic response.

Key Words: admixture mapping, natural selection

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INTRODUCTION

There are four ways that individuals and populations can respond to physiological stress: 1) stress avoidance through cultural practices (including behaviors and technological responses), 2) acclimatization, 3) developmental acclimatization, and 4) genetic adaptation. Often these responses work together synergistically and so can confound a scientific analysis. Carefully designed studies are required to isolate the effects of each of these components of the adaptive process, and are today generally standard in research on mechanisms of physiological response to high-altitude hypoxia.

One area of research that is lagging in the search for mechanisms to explain physiological adaptation to hypoxia is the application of two particular genetic methods: 1) estimating genetic ancestry and admixture mapping and 2) screening for signatures of genetic adaptation. The application of these relatively new genetic approaches shows significant promise for understanding the processes of genetic adaptation to altitude. In this paper we will summarize the ways in which these novel genetic methods can be used to study the genetic regulation of physiological responses to hypoxia. We will also present some new data exemplifying an application of these two approaches.

Genetics, Genics, and Genomics

It is important to consider the basic evolutionary structure of the genome as these conceptions relate to the analytical methods for measuring genetic ancestry, admixture mapping and genetic adaptation. Briefly, admixture is the process by which two or more populations that had been separated join, becoming a new population. Firstly, and somewhat paradoxically, as more genetic markers are combined into an analysis, the results become less and less "genetic". Equation of genetic and genomic information is not always clear-cut and indeed, sometimes diametrically opposed. "Genetic" is the process of inheritance first described by Gregor Mendel who observed that characters were the result of the segregation of "factors" one coming from each parent. Today we call these "factors" alleles and know that they are different forms of genes. Such different forms of a gene are ultimately the result of variability in the DNA sequence comprising the gene often affecting the sequence of amino acids for the protein which the genes codes and other changes that affect the ways in which the proteins are expressed. These variations have distinct effects on the phenotype (or character), some being recessive and others showing dominant effects.

Many traits are like this; the genetic paradigm has been used with tremendous success over the past 100 years to identify more than 1460 traits that are transmitted in single gene, Mendelian fashion (12). All of these traits have distinct genetic effects, meaning they can be observed to segregate in families consistently enough to be mapped onto specific chromosomes. Most of these traits give rise to diseases. It is often the case, however, that not everyone with the risk-allele is affected with the disease; this phenomenon is known as incomplete penetrance. In addition there can be modifying loci that interact with the risk-allele, altering either the chances of showing the disease or its severity. Despite these caveats, genetic traits are those that are inherited in families and show distinct and dichotomous phenotypes.

However, variability in many traits is not the result of one gene (with or without modifying genes), but is instead the result of variation in a number of genes. These traits are called polygenic and do not segregate by Mendelian rules, but instead appear to undergo mixing.

Darwin (and others) described this as blending inheritance. Of course, there are still genes underlying this variability in polygenetic traits (*e.g.* skin pigmentation and stature); it is the combined effect of these genes that results in the continuous nature of the variation in the phenotype. Thus, polygenic traits are properly "genic" – the result of gene action, but not "genetic" in the formal sense. This may seem merely semantic, but there are important conceptual implications inherent in these terms and these distinctions are important for the population genomic paradigm on which surveys for natural selection and ancestry application are founded.

Genomic DNA is a double-stranded molecule composed of sequences of four nucleotide bases (adenine or A, cytosine or C, guanine or G, and thymine or T) organized into chromosomes 10's to 100's of millions of base pairs in length. Genetic markers such as Single Nucleotide Polymorphisms (SNPs) are discrete DNA sequences at specific locations in the genome (*e.g.,* one either has the CC, CT, or TT genotype and these are the only possibilities). SNPs are inherited by the Mendelian rules and so are analogous to simple Mendelian traits. However, as more and more SNPs are combined in an analysis, being in effect (and sometimes literally) averaged together, the result is not "genetic" in either the sense of being able to refer to a distinct phenotype or something that segregates from parents to offspring without blending. Averaging across many markers can be very informative regarding a person's or a population's history. But as more and more markers are added, there is less and less specific information about any one of those markers. (This is of course, unless some subset of the markers is correlated in their distributions across the persons or populations under consideration as is the case in ancestry analysis).

Ancestry Analyses and Admixture Mapping

Specific subsets of genetic markers can be identified which have genetic information regarding particular axes of ancestry (primary indices of genetic covariance) that exist within populations. These markers are most accurately referred to as Ancestry Informative Markers (AIMs) (19) and are basically markers where there are large differences in allele frequency among parental populations. For example, considering that Andeans today have both Indigenous American and European ancestry, a good AIM for Andeans would be one where the C allele of a C/T SNP has an 80% frequency in Europeans and a 20% frequency in Indigenous Americans. The simplest measure of how informative this marker is the delta or allele frequency differential, which in this example is 60%.

By measuring a panel of AIMs it is possible to estimate individual ancestral proportions. Provided there is variation in ancestral proportions across the population (making some persons "more" Indigenous American and others "more" European, for example), one can expect that other markers or groups of markers that are also ancestry-informative will be correlated; that is, differing in the same way across the population. In this way, individual ancestry can be used as a means to study the effects of unknown genes and to identify the locations of these genes, provided they differ between the parental populations (8,18). These associations between AIMs are formed through the process of admixture regardless of the genetic distance between markers or even whether or not they are on the same chromosome. Over time, independent assortment and recombination lead to a decay of associations among distantly-spaced markers. The result is that the level of association observed between an AIM and a phenotype is inversely proportional to the distance between the AIM and the trait gene (6,11).

Population Genetics and Population Genomics

The difference between genetics and genomics is a key point in the new paradigm of population genomics. Population genetics is different from Mendelian genetics in that it is primarily concerned with the behavior of genetic markers and trait-causing alleles in populations, not in families. Population genetics is, fundamentally, evolutionary genetics. It arose out of the "new synthesis" in which evolutionary theory was reinterpreted in the light of the rediscovery of Mendel's work in 1900. Mendelian inheritance is thus part of the foundation of population genetics, akin to an axiom in mathematics, but rarely the object of direct attention. Human population genetics research generally focuses on estimating population parameters that help us model the demographic histories of populations. Questions such as which populations are most closely related, whether particular migrations occurred over short or long periods of time, how admixture has affected contemporary populations, when and how severe were population "bottlenecks" (or reductions in the size of the breeding population) in the past, are the focus of these efforts.

Much human population genetic research has been carried out using mitochondrial DNA (mtDNA) and non-recombining Y-chromosomal (NRY) markers. Since the genes contained in mtDNA and the NRY are passed on intact, from parent to offspring, these markers do not undergo recombination. Thus, all variants are physically connected forever, regardless of the number of base pairs separating them. Since the mtDNA and NRY are very large and highly variable, they are remarkably informative regarding human demographic history. Additionally, since the mtDNA is always inherited through the maternal lineage and the NRY through the paternal lineage, these markers can be used to test hypotheses about differences between female and male migratory patterns and other aspects of mate pairing (13). But despite being very informative (26), mtDNA and NRY studies do not provide more than one measured locus and thus, there is no way to calculate confidence intervals about the point estimates of the parameters that are being studied. Since there is no recombination within the mtDNA and NRY and since these contain a number of genes, adaptation in any of the genes will affect all of the genetic variation and the assumption of selectively neutral evolution (showing no variation in fitness) will not hold.

Another branch of human population genetics has focused on averaging together as many genetic markers as possible to estimate population parameters. The same types of questions that are investigated with mtDNA and NRY markers are addressed with these many-marker averages. One procedural difference between the two methods is that for mtDNA and NRY studies, many more individuals are needed in the population samples compared to studies of large numbers of independent markers. For example, consider two population genetics papers appearing in *Science* a few years ago: the paper by Ke and colleagues focused on NRY analysis in 12,000 Asian men (14), while the paper by Rosenberg and colleagues (20) used average population samples of 20 individuals typed for 377 microsatellite markers.

Population genomics is new branch of population genetics, which is specifically focused on a consideration of both the averages across many markers as well as specific loci individually. A concise summary of this new perspective on genetic variation has been presented by Black and colleagues (3) who describe it as, "the process of simultaneously sampling numerous variable loci within a genome and the inference of locus-specific effects from the sample distributions." Translating simply, population genomics is a model of genome evolution that allows for the analysis of the unique, locus-specific patterns that

result from genetic adaptation in the context of the genome-wide average and largely selectively neural demographic history. Briefly, there are four primary evolutionary forces, 1) mutation, 2) genetic drift, 3) admixture, and 4) natural selection.

Mutation (*i.e.*, a change in the sequence of nucleic acids along the strand of DNA) is the ultimate source of all genetic variation and occurs more or less at random across the genome. Mutation events are unique, occurring in just one chromosome at one particular genomic location, although some nucleotide positions are hypermutable and undergo repeated change. SNPs are an example of those most-commonly occurring types of genetic variation in the genome, and result from the point mutation of a particular base pair.

Genetic drift occurs as the result of segregation of parental alleles in the generation of the current cohort. In a population of infinite size, there is no genetic drift; that is, all alleles present in the parental generation are transmitted to the next. But in the finite population sizes in which persons live, the magnitude of the effect of drift on the genetic variation in the population is inversely proportional to the size of the mating population, increasing as population size decreases. Over time, genetic drift leads both to the loss of some alleles and fixation (*i.e.*, present in all persons) of others, and to genetic differentiation among populations (*i.e.,* populations "drift" apart after separation).

Admixture is the process of gene "flow" or "migration" of alleles from one population to another that occurs when individuals from reproductively-isolated populations interbreed and produce a new population. Admixture reduces the effects of differentiation, making populations more similar. Admixture also creates linkage disequilibrium that can be used for mapping the genes that determine variation in traits that distinguish populations. "Linkage disequilibrium" is the non-random association of alleles at different loci and an important source of statistical power for genetic association testing. Additionally, admixture creates a useful type of genetic structure within populations that might best be called admixture stratification. Admixture stratification can be an important source of information in testing the extent to which variation in a trait across populations is due to genetic differences between the parental populations.

Natural selection, or genetic adaptation, is the process of allele frequency change due to differential fitness (survival and reproduction) of individuals by genotype. Natural selection contrasts with the effects of genetic drift and admixture insofar as only the genetic region around the gene that is under natural selection undergoes change due to natural selection, whereas genetic drift and admixture affect markers across the whole genome. It is notable that, given the random nature of the effects of genetic drift, a substantial amount of variation in level of divergence is expected even though the evolutionary force of drift is applied evenly to all markers across the genome. In other words, drift alone because of the combined effects of many random events will lead to a very wide range in outcomes.

The allele frequency differential (delta), one of the simplest summary statistics, can actually be quite instructive for making comparisons across populations. Delta is calculated as the absolute value of the frequency of allele "C" in population 1 minus the frequency of the "C" allele in population 2 or simply, the difference in allele frequency. Figure 1 shows the histogram of deltas between a sample of Quechua (N=20), a Native Andean population living in Peru, and Nahua (N=20), a Meso-American population living in Mexico (23). The average delta as calculated for total of 11,555 SNPs for these populations is 0.088. This number is smaller relative to that observed when either group is compared to other populations, since these two groups are closely related by both being Indigenous Americans. For

example, when an East Asian sample (N=20; 10 Japanese and 10 Chinese) is compared to the Nahua and Quechua samples, the average deltas are about twice as large, 0.167 and 0.168, respectively. Although the average is 0.088, there is a wide range of delta values for these two populations, with many markers having very low delta levels and a small number showing higher levels. Although, this overall pattern of allele frequency differential is consistent with effects due to genetic drift (akey et al, 2002,), genes that have changed in allele frequency because of the action of natural selection operating after the separation of these populations should show high levels of allele frequency differential. Since the Nahua do not live at high altitude as do the Quechua, it is reasonable to assume that, if genetic adaptation has made high-altitude populations more fit in hypoxic environments, functional alleles should show high delta levels. Thus, the high "tail" of this distribution should contain genes that have undergone genetic adaptation or, in other words, genes near clusters of high-delta markers can be considered *candidate natural-selection genes*.

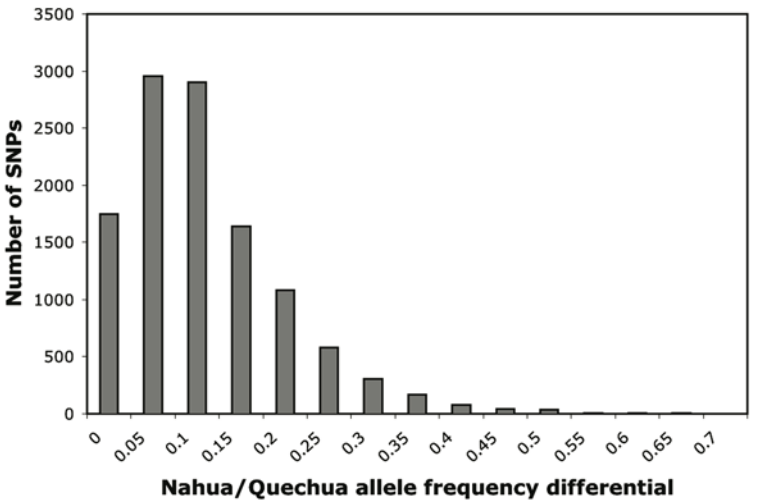

Figure 1. The histogram distribution of allele frequency differential between samples of two Indigenous American populations. The absolute value of allele frequency difference (delta) is shown for Nahua (N=20), a MesoAmerican lowland population, and Quechua (N=20), a Peruvian population from the Altiplano. A total of 11,555 SNPs were analyzed.

Although delta and related measures like F_{ST} (the measure of differentiation when more than two populations are compared), may be informative as to the likelihood that genetic adaptation has occurred near any particular marker in the dataset, more specific tests can be made with a simple modification. The problem is evident since high-delta markers between these two populations could result from either genetic adaptation to altitude (or other aspects of the environment) in the Quechua or to some change in allele frequency due to genetic adaptation in the Nahua. By including a third population, we can compute a more specific measure of differentiation called the Locus-Specific Branch Length (LSBL) as described by Shriver and collaborators (22).

To compute LSBL for three populations (A, B, and C), we use the three pairwise delta

levels (d_{AB}, d_{AC}, and d_{BC}). Then using the three equations below, we can express the branch lengths of the phylogentic tree represented in Figure 2.

1) $x = (d_{AB} + d_{AC} - d_{BC}) / 2$
2) $y = (d_{AB} + d_{BC} - d_{AC}) / 2$
3) $z = (d_{AC} + d_{BC} - d_{AB}) / 2$

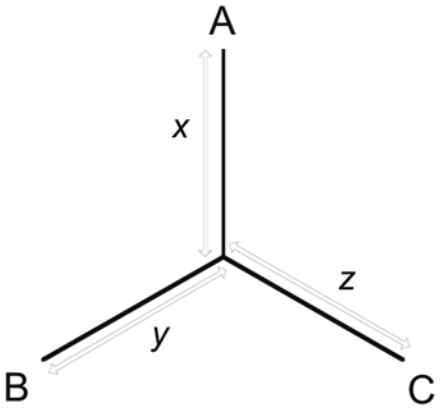

Figure 2. A diagram showing how the Locus-Specific Branch Length (LSBL) is calculated from the three pairwise delta levels. Refer to the text for formulae.

When there are three populations, there is only one topology relating populations and so the only variables are these branch lengths (*x*, *y*, and *z*). Figure 3 shows the distribution of LSBL levels for the three populations, Nahua, Quechua, and East Asian using 11,555 SNPs. As expected in this scenario, the East Asian LSBL estimates are longer, averaging 0.123, than the Nahua or Quechua, averages of 0.0438 and 0.0446, respectively.

The conceptual model of the LSBL is especially consistent with the underlying hypothesis in the case of hypoxic adaptation: we expect that the high-altitude Quechua are going to have adapted at particular loci relative to both the East Asian and Nahua populations. Evolutionary changes will have occurred in each of these populations, but those of greatest interest to hypoxia researchers are the subset of changes leading to high Quechua LSBL. Isolating the changes in one population relative to the two others is a unique facility of the LSBL measure compared to delta or F_{ST} methods. The empirical distributions of LSBL for large number of loci can be used to express the probability of observing LSBL levels of a particular magnitude (17). For example, consider that a locus of interest was measured to have an LSBL value of X. The empirical probability (P_E) is calculated as (# SNPs > X)/(total # SNPs).

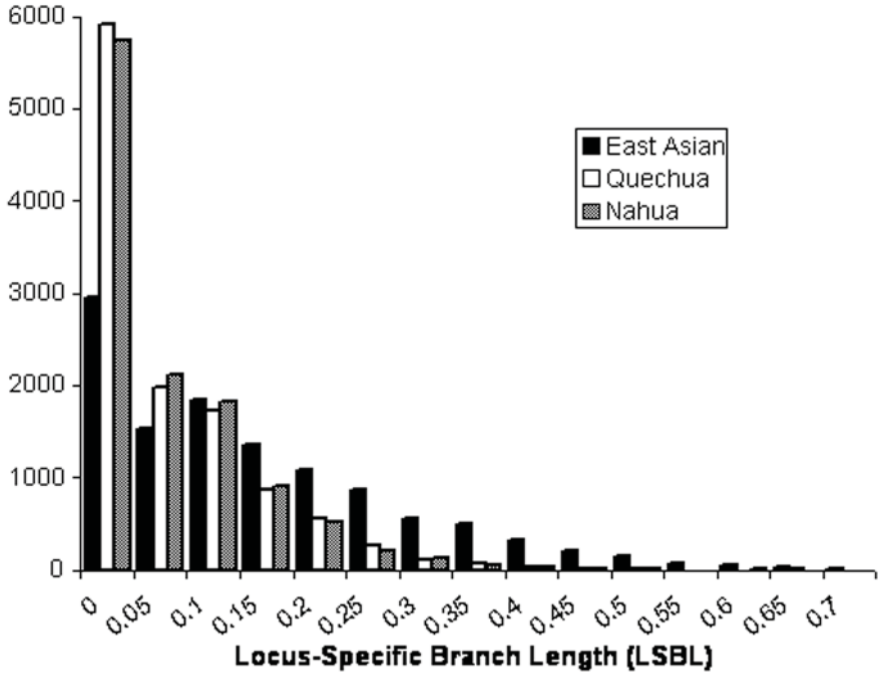

Figure 3. The histogram of the LSBL levels observed across 11,555 SNPs for three populations, Nahua (N=20), Quechua (N=20), and East Asian (N=20; 10 Japanese and 10 Chinese).

We used data generated with the Affymetrix, Inc. (Santa Clara, CA) 10K Mapping Array, a SNP genotyping platform that facilitates the simultaneous analysis of 11,555 SNPs to test a number of candidate genes for evidence of genetic adaptation. We chose four candidate genes of those approximately 40 genes known to contain a "hypoxic response element" or to be involved in the regulation of the hypoxia-inducible factors or HIFs (see Moore in this volume for a fuller explanation). These HIF pathway candidate genes were screened for proximity to any of the 11,555 SNPs. Those candidate genes showing a SNP within 40,000 bp of a SNP are shown in Table 1 along with the allele frequency in the three populations, delta, LSBL and the P_E levels. Five of these results are either suggestive P_E <0.1) or statistically significant (P_E <0.05), the most significant being a SNP near the *EDN1* gene that encodes endothelin where the delta value of 0.275 yields a P_E. = 0.036 and the LSBL value of 0.275 yields a P_E = 0.012.

These results are instructive as to how such studies can be planned and executed. For completeness, one would assay a number of SNPs (five to ten) located within a gene (that is, in either expressed or non-expressed regions embedded within the gene) and compare the deltas and LSBL levels for all of them against the empirical distributions. Genes that show several high-delta or high-LSBL SNPs should then be confirmed as such using independent replicate samples of the three populations. This is especially important when the population sample sizes for SNP markers are small (N<50) as sampling error will decrease the accuracy of allele frequency estimates. Other tests of natural selection, such as sequencing based methods and methods based on Linkage Disequilibrium (LD) can also be used to confirm these population genomic signals.

Table 1. Allele frequencies, delta levels and LSBL levels for HIF Pathway Candidate genes which have nearby SNPs on the Affymetrix 10K Mapping Array .

		Allele frequency						
Gene	**dbSNP rs#**	**Nahua**	**Quechua**	**East Asian**	**Quechua/Nahua delta**	**P_E**	**Quechua branch**	**P_E**
NOS2A	953527	0.588	0.425	0.500	0.163	0.169	0.075	0.226
NOS2A	953528	0.650	0.425	0.525	0.225	**0.074**	0.100	0.157
ADRA1B	952037	0.250	0.100	0.350	0.150	0.202	0.150	**0.093**
EDN1	1321055	0.525	0.800	0.175	0.275	**0.036**	0.275	**0.012**
FLT1	720661	1.000	1.000	1.000	0.000	0.855	0.000	0.490
FLT1	748253	0.900	0.825	0.600	0.075	0.409	0.000	0.490
TGFA	958686	0.250	0.225	0.450	0.025	0.753	0.025	0.421
CUL2	1926554	0.325	0.175	0.200	0.150	0.202	0.025	0.421
NRP2	867344	0.421	0.400	0.600	0.021	0.816	0.021	0.455
NRP2	872943	0.053	0.050	0.150	0.003	0.852	0.003	0.481
NRP1	927099	0.421	0.500	0.368	0.079	0.403	0.079	0.202
EGLN3	1769621	0.625	0.775	0.500	0.150	0.202	0.150	**0.093**
EGLN3	1809461	0.214	0.361	0.250	0.147	0.216	0.111	0.143
EGLN3	958722	0.400	0.425	0.075	0.025	0.753	0.025	0.421

Ancestry Evidence

Our preliminary studies were designed to evaluate the association of Quechua ancestry with hypoxia response traits (4,5). These studies were based on data from 151 young (age 18-35 yrs) Peruvian males and females who were born and raised in Lima, Peru (sea level), and in Cerro de Pasco, Peru (4,338 m). Originally, we used 22 ancestry-informative genetic markers (AIMs) to give estimates of Native American ancestry proportion (NAAP), European ancestry proportion (EAP), and West African ancestry proportion (AAP) for each individual in the study. Recently, as our library of markers has expanded, we have improved the precision of these estimates by using 80 AIMs (unpublished data). These studies have revealed significant associations between NAAP and the decrement in maximal oxygen consumption with altitude exposure ($\Delta\dot{V}O_2max$), the hypoxic ventilatory response to sustained hypoxia (HVR), and the level of resting and exercise ventilation ($\dot{V}_E$) at high altitude. $\Delta\dot{V}O_2max$ was evaluated by measuring the same subjects in both Lima and Cerro de Pasco. After controlling for sea-level $\dot{V}O_2max$, high NAAP was associated with a ~15% smaller $\Delta\dot{V}O_2max$ (4), consistent with earlier studies showing small $\Delta\dot{V}O_2max$ in Andeans compared to European controls (2,9,10,27). However, the ancestry association was only evident in the subset of subjects who also showed large decreases in arterial saturation at maximal exercise at 4,338 m (P=0.03). One possibility is that the ancestry-$\Delta\dot{V}O_2max$ association is easiest to detect in well-trained or highly motivated subjects who are more likely to experience pulmonary gas exchange limitation and/or exercise induced hypoxemia during exercise, particularly at altitude. Even stronger ancestry associations were detected with the HVR measured at sea-level and the altitude $\dot{V}_E$(unpublished data). NAAP was inversely related to the HVR measured after 10 minutes of isocapnic hypoxia given at sea-level (R=-0.36,P=0.04). NAAP was also inversely related to exercise $\dot{V}_E$ (R=-0.50, P=0.005) when subjects were tested at altitude in Cerro de Pasco. Thus, Quechua ancestry may be partly responsible for the well-known hypoxia tolerance of Andeans (i.e., small $\Delta\dot{V}O_2max$), their "blunted HVR" (7,17,16,21,24), and their relative exercise hypoventilation compared to European controls.

SUMMARY AND FUTURE DIRECTIONS

Given high-altitude native populations have undergone genetic adaptation making them better fit under hypoxic stress, two methods of genomic analysis can and should be used to identify the particular genes involved. The two methods, 1) Estimating genetic ancestry and admixture mapping and 2) Screening for signatures of genetic adaptation provide complementary information. Screening for genetic adaptation can be carried out on a genomic scale when sufficient numbers of genetic markers are available (25). It is unclear the number of genetic markers that will be required to properly tag the entire genome, but this number is likely to be quite high (> 100,000 evenly spaced evolutionarily informative SNPs). DNA sequence information can also be used to test for genetic adaptation on a candidate locus level now and within 5 to 10 years, on a genome-wide level as the costs of sequencing come down. The population genomic paradigm provides an appropriate context within which to identify hypoxia genes, but further theoretical and analytical developments are required to realize the full potential of these methods. Additionally, there is the need for a much more complete understanding of the demographic histories of South

American populations.

ACKNOWLEDGMENTS

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Chapter 10

AN EVOLUTIONARY MODEL FOR IDENTIFYING GENETIC ADAPTATION TO HIGH ALTITUDE

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Abstract: Coordinated maternal/fetal responses to pregnancy are required to ensure continuous O_2 delivery to the developing organism. Mammals employ distinctive reproductive strategies that afford their young an improved chance of survival through the completion or the reproductive period. Thus, mortality prior to the end of the reproductive period is concentrated in the earliest phases of the lifecycle. At high altitude, fetal growth restriction reduces birth weight and likely compromises survival during the early postnatal period. Population variation in the frequency of the altitude-associated increase in intrauterine growth restriction (IUGR) demonstrates that multigenerational Tibetan and Andean high-altitude populations are protected compared with shorter duration, European or Han (Chinese) residents. This experiment of nature permits testing the hypothesis that genetic factors (a) influence susceptibility to altitude-associated IUGR, (b) act on maternal vascular adjustments to pregnancy determining uteroplacental blood flow, and (c) involve genes which regulate and/or are regulated by hypoxia-inducible factors (HIFs). Serial, studies during pregnancy as well as postpartum in Andean and European residents of high (3600 m) and low (300 m) altitude will permit evaluation of whether uteroplacental O_2 delivery is lower in the European than Andean women and, if so, the physiological factors responsible. Comparisons of HIF-targeted vasoactive substances and SNPs in or near HIF-regulatory or targeted genes will permit determination of whether these regions are distinctive in the Andean population. Studies coupling genetic and genomic approaches with more traditional physiological measures may be productively employed for determining the genetic mechanisms influencing physiological adaptation to high altitude.

Key Words: adaptation, hypoxia, hypoxia inducible factor (HIF), IUGR, natural selection, preeclampsia, uteroplacental ischemia

Hypoxia and Exercise, edited by R.C. Roach *et al.*
Springer, New York, 2006.

INTRODUCTION

Studies of human physiological adaptation to high altitude (defined here as >2500 m or 8000 ft) have long sought to determine whether or not there are genetic factors involved. Such efforts have been hindered by the inherent difficulties in distinguishing between genetic attributes *vs*. those that are acquired as a result of prenatal, postnatal, or later-in-life influences (9). In the language of geneticists, the phenotype (P) is a function of genetic (G) and environmental (E) influences plus the interactions between them (G x E); rarely, are only genetic factors responsible for visible traits. Interaction is so ubiquitous that some have questioned whether any influence is purely genetic or environmental (47). Such interactions include the influences of age, nutrition, disease or other kinds of environmental characteristics on the expression of genetic traits. For example, at high altitude, the larger lung volumes of lifelong, Andean high-altitude residents reflect both developmental exposure and a hereditary potential for larger lung dimensions (19). In addition. Brutsaert and co-workers have shown that the extent of an individual's physical fitness -- largely a function of acquired traits such as habitual exercise levels and training -- influences the extent to which genetic factors confer protection from an altitude-associated decrement in maximal exercise capacity (8).

The purpose of this article is to describe a model for identifying the genetic and/or genomic contribution to human adaptation to high altitude. Specifically, this model lays the groundwork for applying the analytical techniques described in this volume by Shriver *et al.,* to the adaptive challenge of fetal growth restriction posed by residence at high altitude. For reasons elaborated upon below, because the risk to survival prior to the end of the reproductive period is greatest during the period of pre- and early post-natal life, we elect to focus on fetal growth restriction as our index of high-altitude adaptation. Since genetic changes occur over generations and hence require long periods of time, we also choose to compare populations with and without multigenerational exposure to high altitude, while proposing to make provision for controlling for differences between groups unrelated to high-altitude exposure.

DEFINING ADAPTATION

From a genetic perspective, evolution can be defined as change in gene (allele) frequency over time. Four factors are involved– mutation, genetic drift, gene flow and natural selection – but only one of these, natural selection, is directional. Natural selection is also the force of greatest interest to physiologists since, generally, it is the effects of genes on the organism's ability to adapt to the environment that is of paramount concern. "Adaptation" in an evolutionary context refers to the ability to live and reproduce in a given environment (17). In principle, adaptations can be grouped into those affecting fertility (the production of live offspring) and mortality (in an evolutionary context, the ability of the organism to survive until the end of its reproductive period). "Fitness" is the net result of influences on both fertility and mortality, sometimes simply referred to as reproductive success. Distinguishing between adaptations affecting fertility and mortality can be difficult, especially in mammals that, as a group, shelter their young within their mother's uterus, making early mortality difficult to detect.

Mammalian distinctions. The appearance of mammals in the paleontologic record changed natural selection from operating by an "r"- to a "k"- strategy. An "r"-based strategy is characterized by the production of a large number of young, each of which has a low probability of survival. The "k"-strategy employed by mammals relies upon the production of fewer eggs, each of which has a greater probability of being fertilized and, if fertilized, a greater probability for survival. In short, the changeover from an "r" to a "k" strategy reflects a shift from a more "quantity" to "quality" reproductive approach. Still, among mammals, probably only on the order of 20% of fertilized eggs survive until birth.

A prolonged intrauterine period greatly enhances the likelihood of survival but poses a different kind of challenge; how to house a 50% genetically dissimilar organism without the mother's immune detection and rejection? For some mammals, the answer is to protect the offspring by having a "shell" (more a leathery sort of skin) to protect the offspring from immune detection while in utero. These are "prototherian" mammals, exemplified by the duck-billed platypus. Another strategy is that employed by "metatherian" mammals, like kangaroos or wallabys, which give birth early to an extremely immature offspring that is housed in the mother's pouch where it can nurse and grow to a large enough size to be able to fend for itself. The metatherian strategy appears to have been the kind employed by the earliest mammals (105). "Eutherian" mammals' reliance on the placenta carries the "r" *vs.* "k"-approach a stage further insofar as the placenta greatly improves the conceptus' chance of survival by permiting a period of prolonged gestation for fetal growth and development. The placenta is a specialized tissue of fetal origin that not only aids in nourishing the fetus but also minimizes its detection by the mother's immune system[1].

Shape of the age vs. survival curve. The influence of the placenta on the shape of the survival curve is illustrated in Figure 1. Defining life as beginning at conception[2], mortality rates in fish are very high during hatching and then decline, as they get larger and are better able to flee from predators. The protection afforded by shelled eggs lowers mortality somewhat during early life. Protection is further enhanced in placental mammals, displacing the survival curve to the right even more. Relatively high mortality soon after conception is still present in placental mammals, likely due to implantation failure, chromosomal or other kinds of genetic abnormalities which are nearly always lethal and result in spontaneous abortion. An improved chance of survival following this early, prenatal period is carried to an extreme in humans whose smaller number of births, extended period of infancy, and social support systems reduce infantile and childhood mortality relative to other mammals. Even more remarkably in humans, survivorship extends beyond the end of the reproductive period into old age.

One point requires clarification: the difference between "lifespan" and "life expectancy." Sometime used interchangeably, "lifespan" refers to a probably, genetically-endowed maximum length of life for a given species, whereas "life expectancy" is the probability that a given organism will achieve this lifespan. For humans, the lifespan is estimated as being approximately 120 years. Life expectancy is generally calculated at birth but can, in principle, be calculated at any age. When calculated at birth, by far the greatest contributor to life expectancy is the mortality rate during infancy and childhood. If calculated at age 15, life expectancies are quite similar even when comparing societies with markedly different standards of living (21).

The point here is that humans follow a reproductive strategy in which mortality, prior to the end of the reproductive period, is concentrated in the earliest phases of the lifecycle.

In other words, once born and especially once infancy and childhood are completed, the chances of surviving through the reproductive period are very high. In relation to high altitude, the implication here is that the most important determinants of survival are likely to be those affecting intrauterine mortality or mortality soon after birth. Fertility does not seem to be adversely affected at high altitude (103); on the contrary, levels appear to be higher in some high- than low-altitude Peruvian groups (26). Therefore, the most important influences of altitude are likely to be centered on fetal growth and/or gestational age.

Importance of birth weight. For humans, the single most important predictor of neonatal mortality is small size at birth, whether due to fetal growth restriction or preterm delivery (52). This is illustrated by the continuing decline in mortality rates from the neonatal through the infant and childhood periods in Figure 1.

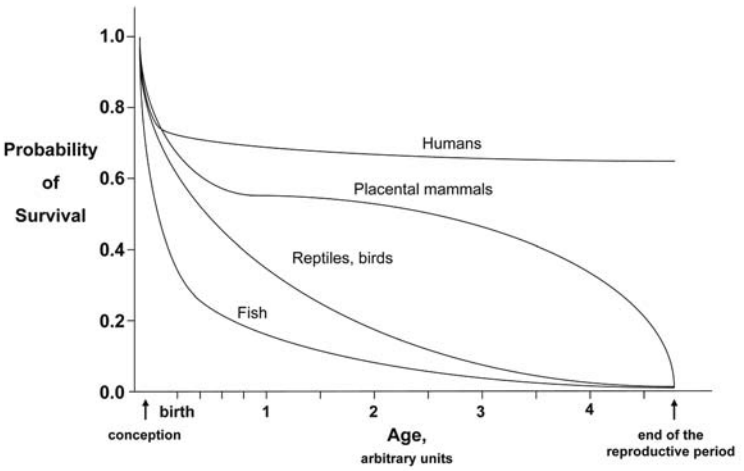

Figure 1. For fish and reptiles or birds, mortality is especially high following hatching and then gradually declines, with all individuals dying after the completion of reproduction. Placental mammals, initially, have high intrauterine mortality but an improved chance of survival relative to fish, reptiles or birds. Mortality gradually declines following birth for most placental mammals, with all deaths occurring by the end of reproduction. Humans have a distinctive pattern in which mortality is relatively low during infancy and childhood and then very low during adolesence and the adult years, with a considerable proportion surviving after the completion of the reproductive period. This graph is drawn for illustrative purposes and is not strictly to scale. Actual age-specific mortality risks vary by historical period and by society.

The contributions of gestational age and birth weight to neonatal mortality vary, depending on the particular ages and weights involved, as shown by the non-symmetric shape of the mortality boxes in the fetal growth charts first advanced by Dr. Lula Lubchenco nearly 50 years ago (Figure 2). The development of such charts, now used the world over, permits identifying whether or not a given newborn is at "risk", it ranks as one of the great public health advances of our times. Sociocultural factors also influence the effects of fetal growth and gestational age on neonatal mortality; preterm births are less likely to occur in developed countries where medical technology permits delaying labor or hastening maturation of the fetus' lungs when labor is threatened, making IUGR an especially important factor

(28). But today in the developed as well as the developing world, as well as throughout human existence, a high proportion of infant mortality is likely attributable to the influences of fetal growth restriction and preterm delivery.

While many factors contribute to IUGR[3], one of the more pervasive is uteroplacental ischemia. As elaborated below, uteroplacental blood flow increases more than 30-fold during pregnancy, fueling the exponential rise in fetal growth, and making any reductions in blood flow potentially important. While sometimes not thought of as a nutrient, oxygen can be so considered since oxygen, like other nutrients, is a source of energy (ATP supply) as well as a cofactor in numerous metabolic processes. Other nutrients required for fetal growth include glucose, amino acids, various trace minerals and vitamins.

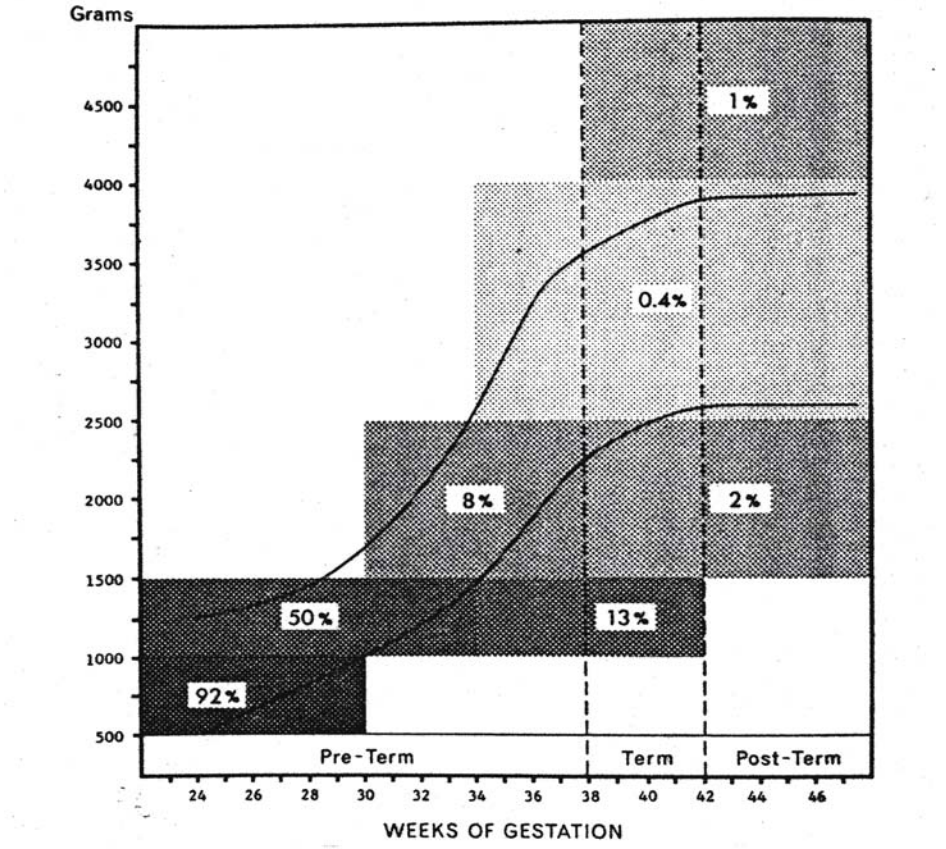

Figure 2. Birth weight (gm, y axis) and gestational age (x axis) influence neonatal mortality, shown as the percent deaths during the first 28 days of postnatal life. Mortality declines with advancing gestational age and increasing birth weight. The figure is reprinted from Lubchenco (52) who compiled data for all University of Colorado Medical Center newborn admissions from July 1, 1958 - July 1, 1968.

EFFECT OF HIGH ALTITUDE ON BIRTH WEIGHT

Epidemiological, anthropological and public health observations. High altitude occupies a special place in the history of investigating the cause(s) of low birth weight (conventionally defined as <2500 gm or 5.5 lbs). Until the 1970s, the World Health Organization and other such agencies considered any low birth weight baby to be "preterm" since gestational age information was not routinely sought. The first observations that hypoxia slows fetal growth on a population level were made at high altitude (49). In these studies, the possibility of shortened gestation was carefully considered; popular opinion held that the lack of snowplows prevented women from reaching the hospital and hence doctors delivered their patients early (65). The actual data, however, showed that gestational age did not differ from low-altitude values. Hence fetal growth restriction was identified as the primary cause in this early, as well as numerous more recent, studies (25, 37, 73). From ultrasound studies and altitude comparisons across a range of gestational ages, growth measurably begins to slow about week 30 (43, 102).

The birth weight decline averages 100 gm per 1000 m altitude gain (37). The effects of altitude are greater than those of low maternal weight gain, smoking, primiparity or preeclampsia and second only to gestational age in importance (37). Given the more than 100,000 Coloradans and 140 million persons living at high altitudes worldwide, high-altitude residents are the single largest group at risk for IUGR (41). The primary factor responsible appears to be the hypoxia associated with residence at high altitude (25, 49, 73). Additional contributors are the altitude-associated increase in preeclampsia, which accounts for about half the birth weight decline, as well as yet to be fully elucidated interactions with other factors such as smoking (37, 39, 55, 66, 78, 81).

While occurring worldwide, the magnitude of the altitude-associated birth weight reduction varies in relation to the duration (in generations) of high-altitude exposure. That is, Tibetans and Andeans who have lived at high altitudes for 10,000+ yrs show one-third the reduction present in European or Han ("Chinese") populations that have resided at high altitudes for <500 years (79). Such variation does not appear attributable to maternal body size, nutrition, health care or birth weight present at sea level (29, 64). Nor do birth weights of babies born to lifelong high-altitude residents differ from those of babies born to women moving to altitude as adults, suggesting that developmental effects are not chiefly responsible (30, 69, 106).

Given the lower birth weights present at high altitudes, the expectation would be that neonatal and, by extension, infant mortality would be increased as well. Consistent with this, Bolivia and Peru have the highest infant mortality rates in South America and, in the case of Bolivia, the highest in the western hemisphere (80). There is a proportional rise in mortality with increasing altitude within Bolivia for both urban and rural dwellers, with rural rates being higher than those seen in urban areas (7, 24). Since there is considerably greater health care access in urban than rural regions, the urban-rural differences support the importance of health care for the mortality rates observed. But since the altitude rise is present for both urban and rural residents, the data also suggest that altitude is a likely contributor. In Colorado, infant mortality was greater in the high *vs.* low-altitude regions of the state until about 1980. With the advent of specialized neonatal care services and transport systems, mortality rates fell, particularly at the higher altitudes, so that now rates are similar throughout the state or in comparison to national levels (49, 62, 102, 109). There is

continuing controversy as to whether the mortality risk for a given birth weight is modified by altitude. Recent Colorado or USA data indicate a slightly lower birth weight specific mortality risk at high than low altitude (102, 109). This may, in turn, be due to the more chronic nature of the altitude-related reduction in birth weight or to evidence supporting a greater utilization of specialized health care services by the higher-altitude women (102). The lack of a vital statistics system or gestational age data in South America, Tibet, or Ladkakh prevents assessing birth weight specific mortality risk in these regions. Nonetheless, low birth weight babies have higher mortality than do their normal weight counterparts -- not only during infancy but possibly at later ages as well (4) -- suggesting that it is not good to be "small" at high (or any) altitude.

Maternal oxygen transport adjustments to pregnancy. Pregnancy alters all the components of the maternal O_2 transport system; of these, greater uteroplacental blood flow is the most important. Arterial O_2 tension rises, due to an increase in ventilation that, in turn, reflects the central as well as peripheral, stimulatory effects of progesterone and estrogen hormones and elevated metabolic rate (68). But at low altitude, this ventilation rise does not normally influence arterial O_2 saturation since values are already nearly maximal. Blood volume expands, with the rise in plasma volume exceeding that of red cell mass, such that both hemoglobin concentration and arterial O_2 content decline. Thus increased uteroplacental O_2 delivery depends entirely on greater uteroplacental blood flow. A rise in uteroplacental blood flow is accomplished by uteroplacental as well as non-uteroplacental, systemic means. There is an early fall in systemic vascular resistance (SVR) that, together with the increase in blood volume, raises resting cardiac output some 40% (11). The fall in SVR is likely due to vasodilatory effects of increased NO production, decreased sympathetic tone, and a fall in the potent vasoconstrictor endothelin-1 (ET-1) (27, 53).

In the uterine circulation, an anastomosis develops between the ovarian branch and the main uterine artery (UA), providing the uterus with dual, bilateral arterial blood supply. Because the fall in uteroplacental vascular resistance is greater than that which occurs in the rest of the systemic circulation, a large fraction of the increased cardiac output is directed to the uteroplacental vascular bed. Most (~80%) goes through the two UA, raising unilateral flow from ~10 ml/min in the non-pregnant state to ~350 ml/min near term (82). This impressive rise in UA blood flow is due both to a doubling of intraluminal diameter (which quadruples cross-sectional area) and to increased blood flow velocity. UA vasodilation, greater distensibility as well as growth in all layers of the vessel wall are responsible for the increase in UA diameter (13). Similar changes likely occur in the mesometrial and basilar arteries, but the mechanisms by which these changes interact with the trophoblast invasion occurring downstream (at the level of the spiral arterioles) are not well understood.

An important distinction arises between species with hemochorial *vs.* epithelioral placentas (*i.e.,* humans, most other primates, rodents, and guinea pigs *vs.* sheep, dogs, cows, etc) in terms of the vessels constituting the primary site of uteroplacental vasculature resistance. In hemochorial species, trophoblasts invade approximately one-third of the way through the uterine wall, whereas trophoblast invasion is only one cell layer thick in epitheliochorial species. As a result, in hemochorial species the vessels determining uteroplacental vascular resistance reside largely outside the uterus; more precisely, 2/3^{rds} of uteroplacental vasculature resistance is located in the mesometrial, main UA and ovarian arteries with only 1/3rd being located in uteroplacental channels (63, 96). This is the opposite of that which occurs in species with epitheliochorial placentas where the uteroplacental vessels

comprise the major site of vascular resistance. Our studies have focused on the UA since it makes a demonstrable contribution to uteroplacental vascular resistance in the hemochorial species under study and can be visualized in humans.

Mechanisms by which chronic hypoxia may alter uteroplacental oxygen delivery. While in principle, the lower birth weights at high altitudes could be due to alterations in maternal and/or placental characteristics; we suspect maternal characteristics for several reasons. First, in a limited number of women in which we were able to make measurements of both UA diameter and blood flow velocity, UA volumetric blood flows were approximately one-third lower in near term women residing at high (3100 m) *vs*. low (1600 m) altitude in Colorado (112). Second, we had previously noted that not only were birth weights lower but also the maternal complication of preeclampsia was more common at high altitude. This was an accidental outgrowth of noting a higher than expected occurrence of preeclampsia in the high-altitude residents being studied physiologically. When we compared our sample to all women delivering during the study period, we found a surprisingly high incidence of preeclampsia in the population at large (67). Later, in a more systematic study, we confirmed the increased incidence of preeclampsia at high altitudes (81). Subsequent studies have replicated this finding elsewhere (39, 54). Third, placental characteristics have not, to date, been shown to be markedly different at high *vs*. low altitude, although generalized thinning has been reported (58). TissotvanPatot *et al.,* found a smaller proportion of maternal vessels had undergone trophoblast invasion and converted to a low-resistance circuit. But a greater absolute number of vessels meant that the number of "converted" vessels was the same at low and high altitude (101). Further studies are greatly needed to indicate whether or not metabolic processes are altered in potentially significant ways in high *vs*. low altitude placentas.

At high altitude, the pregnancy-associated increase in ventilation raises arterial O_2 saturation. Near-term O_2 content still falls due to greater plasma volume than red cell mass expansion (69) (61). While the extent to which the rise in ventilation is able to defend O_2 content relates positively to infant birth weight (69), heavier birth weights in long term high-altitude natives are not due to differences in arterial oxygenation since Tibetans and Andeans do not have higher arterial O_2 content than their Han or European counterparts (71). Thus, UA blood flow is likely to be the key determinant of uteroplacental O_2 delivery at high as well as at low altitude.

Non-uteroplacental as well as uteroplacental factors may be responsible for lowering uteroplacental O_2 delivery at high altitude. Cardiac output is lower, probably as the result of lower blood volume and/or higher SVR (40). The higher SVR may be due, in turn, to increased vasoconstrictor and/or reduced vasodilator production. Acute (hours) and more chronic high-altitude exposure raise sympathetic nervous system activity and catecholamine levels in non-pregnant women (60). ET-1 levels are also elevated by chronic hypoxia (72) and in preeclampsia (20). Underscoring the potential importance of ET-1, ET-1 A receptor blockade prevents the IUGR as well as the accompanying reduction in uteroplacental blood flow that is associated with hypoxia as well as NO synthase blockade (99). A third possibility -- reduced systemic NO production -- is not strongly supported by current data (108).

Concerning the uteroplacental circulation, UA blood flow near term is one-third lower in Colorado residents of high (3100 m) *vs.* low (1600 m) altitude, due to less pregnancy-associated increase in UA diameter (111). UA diameters and volumetric flows are also lower

in Han residents of high- *vs.* low altitude (12) and in our preliminary studies of European *vs.* Andean residents of high altitude (70). Our data, like those of Krampl and co-workers (42), do not indicate that the lower UA blood flows are due to higher indices of uteroplacental vascular resistance, suggesting that the factors limiting uteroplacental blood flow reside in the uterine, not primarily the placental, vessels.

Our experimental animal studies demonstrate that chronic hypoxia opposes the normal pregnancy-associated changes in the UA. During normoxic pregnancy, the UA demonsterates an increased vasodilator response to flow; this enhanced flow vasodilation is absent in UA from chronically hypoxia animals (57). Similar findings are reported in preeclampsia, where a failure of mesometrial artery flow vasodilation also occurs (44). Chronic hypoxia also inhibits UA growth such that there is only half as much rise in DNA synthesis in vessels isolated from chronically-hypoxic *vs*. normoxic animals (88). Both these flow and growth alterations may stem from a lack of pregnancy-associated increase in NO. Chronic hypoxia reduces NO-dependent vasorelaxation to acetylcholine in isolated guinea pig UA rings and inhibits the pregnancy-associated increase in endothelial NO synthase protein (NOS III) in whole vessel homogenates (107). NOS III is also known to be important for the hypertrophic outward vascular growth characteristic of pregnancy (74). Unknown is whether hypoxia also affects the pregnancy-associated alterations in other vasodilators (*e.g.,* endothelial-derived hyperpolarizing factor), growth factors (*e.g.,* VEGF, PGF) and vasoactive factors (*e.g.,* ET-1, catecholamines), although our preliminary data and limited reports in non-pregnant persons (56) suggest that it does. In contrast, in sheep which did *not* exhibit altitude-associated reductions in fetal growth or UA blood flow (40) chronic hypoxia prompts an *exaggerated* increase in vasodilator response to acetylcholine and a greater rise in NO production, NOS III protein and message than seen during pregnancy at low altitude (113). Likewise, whereas chronic hypoxia did not alter the pregnancy-associated fall in UA vasoconstrictor response to phenylephrine in the guinea pig, this was augmented in sheep UA as a result of decreased alpha1-adrenergic receptor density, binding affinity and inositol phosphate 3 production (35). Such species variation in the effects of chronic hypoxia supports an important role for genetic mechanisms in the regulation of UA vascular response to pregnancy.

THE ROLE OF THE HYPOXIA-INDUCIBLE FACTOR (HIF) SYSTEM

HIF and O_2 homeostasis. HIF plays a central role in O_2 homeostasis, regulating over 70% of hypoxia-responsive genes. Thus HIF-regulated pathways are logical targets on which selection for traits influencing susceptibility to hypoxia-related disorders could be expected to act. The molecular mechanisms by which such regulation is achieved have been the subject of intense investigation (Figure 3). HIF is a highly-conserved heterodimer consisting of class one molecules -- the constitutive HIF1beta/ARNT complex -- and one of three class two molecules -- HIF 1, 2 or 3alpha. Despite continual production, degradation is sufficiently rapid that the HIFalpha proteins are virtually undetectable in normoxia. This degradation requires trans-4-hydroxylation at proline-564 and -402, recognition and binding by the VHL protein, ubiquination by a E3 ubiquitin ligase complex (consisting elongin C/elongin B, cullin 2, and the RING-H2 finger protein Rbx-1), and transport to the

proteasome (89). But under hypoxia and selected other circumstances (*e.g.,* specific oncogenes, PHD enzyme inhibition, presence of large divalent metal ions or iron chelators), HIFalpha escapes hydroxylation and recognition by VHL. This permits HIF protein levels to rise, translocate to the nucleus, heterodimerize, and transcriptionally activate genes containing the cis-acting HRE 5'ACGTG(C/G)3' (93).

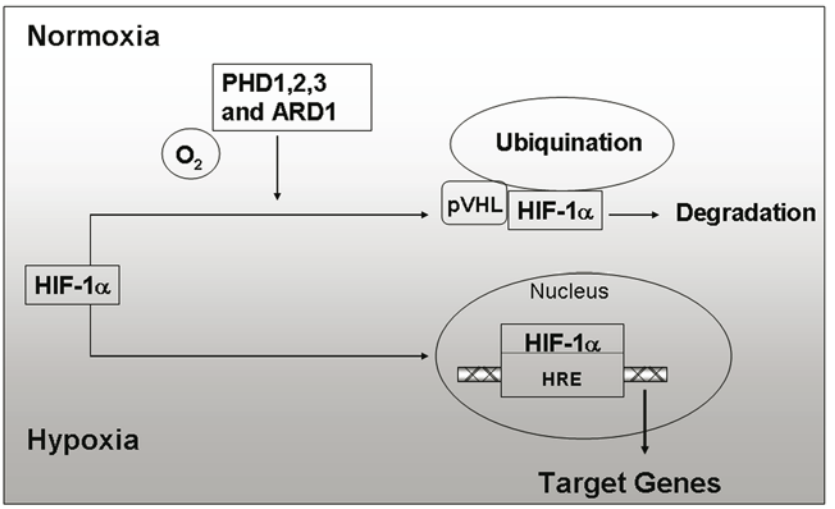

Figure 3. HIF-1 alpha regulation (schema courtesy of Kurt Stenmark).

Mechanisms and evidence of action during pregnancy. Over 40 HIF-regulated or regulatory genes have been identified whose functions influence the vascular adjustments to hypoxia and pregnancy (85; Table 1). Moreover, variation occurs in these genes and such variation alters circulating plasma levels (86). Together with previous reports demonstrating that susceptibility to preeclampsia as well as IUGR is heritable (2, 36, 50, 51, 90, 104), there is a powerful rationale for screening these genes in an effort to find those responsible for the population variation in hypoxia-associated IUGR. Additional, direct support for the involvement of HIF-regulated genes comes from recent studies showing that trophoblast cells from preeclamptic placentas over express HIF-1 and 2 alpha (10, 84). Their transcriptional targets such as ET-1 and VEGF are also likely to be altered, since both have been implicated in the characteristic vasoconstriction, endothelial damage and reduced uteroplacental blood flow of preeclampsia (18, 20, 46, 59, 95, 97-100). Vascular growth and remodeling are also likely affected; hypoxia, both in vitro and in vivo, alters the production of cytokines and other growth-related factors required for trophoblasts to transition to the invasive phenotype and remodel maternal spiral arterioles (22, 23, 101).

A genetic and genomic strategy for identifying variation in HIF-targeted or regulatory genes. As noted above, long-term (multigenerational) residents are relatively protected from the altitude-associated increase in IUGR and such protection appears due to ancestry-dependent, perhaps genetic, factors that permit normal maternal vascular adaptation to pregnancy. Thus, high-altitude populations provide a natural laboratory for determining whether genetic factors influence susceptibility to IUGR and possibly other conditions

characterized by uteroplacental ischemia and, if so, the pathways or mechanisms through which such genes act.

Logical next steps involve a search for variation in HIF-regulatory and -regulated genes, and linking such variation to the maternal vascular abnormalities in IUGR and preeclampsia. Our concept here is that genetic variation in these systems exists in all populations and may explain, in part, susceptibility to preeclampsia and IUGR. High-altitude residents are expected to have been acted upon by natural selection across many generations and thus to preferentially express those genetic variants conferring resistance.

The availability of single nucleotide polymorphisms (SNPs) from the Human Genome Project provides a crucial methodological tool. SNPs permit evaluating the contribution of multiple genes to disease susceptibility in conjunction with varying environmental exposures. SNPs have been described in the HIF-regulated genes listed in Table 1, including proteins of known functional significance during pregnancy such as ET-1, VEGF, tyrosine hydroxylase (the rate-limiting step in catecholamine synthesis) and glucose transporters (31, 32, 34, 76). SNPs also occur in HIF-regulatory genes. This is exemplified by VHL, a tumor suppressor gene, which contains SNPs in coding regions that influence whether the person develops phaeochromocytoma, hemanigoblastoma, or renal cell carcinoma (14). Such SNPs are functional insofar as when VHL is mutated at specific amino acids and expressed in mammalian cells, it can no longer interact with HIF1alpha and send it to the proteasome for degradation.

Genomic approaches have been infrequently applied to pregnancy complications but offer considerable power for identifying genetic involvement in complex diseases. Thus they are increasingly being used in studies of human genetic variation to provide information unavailable from other approaches (5, 91). Such genomic approaches stem from the recognition that since geographic separation of human populations is quite recent[4], most genetic variation is shared among all populations (48). Thus, we expect low levels of genetic differentiation across most genes but, given our overall hypothesis, greater divergence in the Andean population in those genes that are HIF-regulated or -regulatory. Such differences in divergence are readily detectable using statistically sophisticated methodologies as further described by Shriver et al. in chapter 9 of this volume.

SUMMARY AND CONCLUSION

The reduced O_2 availability of residence at high altitude restricts fetal growth and increases the frequency of preeclampsia, making high-altitude residents the single largest group at risk for these disorders of pregnancy/fetal life. The altitude-related increase appears due, in part, to alterations in maternal vascular reactivity, growth and remodeling that reduce uterine artery blood flow. Moreover previous studies demonstrate that multigenerational compared with shorter-term high-altitude residents are protected from the altitude-associated increase in IUGR, probably as the result of greater UA blood flow. Based on recent evidence demonstrating that hypoxia-inducible transcription factors (HIFs) play a central role in regulating O_2-sensitive genes, are implicated in pregnancy disorders, and our preliminary evidence that they are differentially regulated in long- *vs.* short-term populations, future studies will test the overall hypothesis that genetic variants in HIF-targeted or regulatory pathways protect multigenerational high-altitude residents from hypoxia-as-

sociated IUGR. Strategies that couple genetic and genomic approaches with more traditional physiological tools may permit the identification of genes influencing physiological responses to pregnancy and can be productively employed for investigating other health effects of high altitude as well. Such studies are relevant not only for the 140 million high-altitude residents worldwide, including more than 100,000 in Colorado, but also the larger number of persons whose health is compromised by hypoxia.

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[1] While the placenta has been subject to intense investigation, surprisingly little attention has been devoted to the comparative physiology of the placenta or its potential influences on mammalian evolution; topics worthy of future exploration.

[2] The ongoing debate concerning abortion demonstrates that the definition of "life" is subject to cultural, as well as biological interpretations.

[3] Birth weights

Chapter 11

HYPOXIC PRECONDITIONING AND ERYTHROPOIETIN PROTECT RETINAL NEURONS FROM DEGENERATION

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Abstract: Reduced tissue oxygenation stabilizes the alpha-subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1). This leads to the induction of a number of hypoxia responsive genes. One of the best known HIF-1 targets is erythropoietin that exerts neuroprotective effects on ischemia-related injury in the brain. Thus, pre-exposure to low environmental oxygen concentrations might be exploited as a preconditioning procedure to protect tissues against a variety of harmful conditions. We present recent work on neuroprotection of retinal photoreceptors induced by hypoxic preconditioning or by systemically elevated levels of Epo in mouse plasma.

Key Words: hypoxia-inducible factor-1, apoptosis, photoreceptor, blinding disease, overexpression of EPO

INTRODUCTION

Reduced oxygenation triggers several adaptive responses in mammals including man. At the cellular level, low oxygen supply induces expression of a variety of oxygen-regulated genes such as erythropoietin (Epo), vascular endothelial growth factor (VEGF) and several glycolytic enzymes. Once 'loaded' with the products of hypoxically activated genes, the organism is able to cope with hypoxia. Expression of oxygen-dependent genes is transient and the return to normoxic conditions will gradually reduce the intra- and extracellular concentrations of induced proteins to pre-hypoxic levels. Upon re-oxygenation, however,

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there is a given period of time in which the tissue is still 'loaded' with gene products that were upregulated during the antecedent hypoxic period. If re-exposed to hypoxia, these tissues are better protected against hypoxic injury compared to non-preexposed ones. We call this concept *hypoxic preconditioning* (Grimm et al; submitted).

It is conceivable that hypoxic preconditioning of cells, tissues, animals or even patients has a clinical potential (reviewed in (16)). We propose that the full set of oxygen-dependent genes will include some that can provide tissue- or cell-protective effects. This protection may not be limited to hypoxic/ischemic insults but may also protect against other stimuli that otherwise might harm an organism. It is therefore desirable to identify such protective genes or gene products that have a potential as therapeutical drugs. We show that hypoxic preconditioning increases the resistance of neuronal tissue against harmful insults and that Epo is one of several putative factors involved in this protection.

THE MOLECULAR RESPONSE TO HYPOXIA IS VERY FAST

Many reviews on the oxygen-sensing mechanism and the involved hypoxia-inducible factors and prolyl hydroxylases have been published recently (24, 25, 53, 54). In brief, hypoxic exposure of cells leads to the accumulation of the α-subunit of the hypoxia-inducible factor-1 (HIF-1). Heterodimerization of HIF-1α with its partner HIF-1β (also termed ARNT for arylhydrocarbon receptor nuclear translocator) leads to the formation of the HIF-1 complex that binds to the hypoxic response element that is present in all HIF-target genes described so far (3) and enhances their transcription. While hypoxia quickly stabilizes HIF-1α protein, normoxia leads to immediate degradation of HIF-1α (28). Stabilization of HIF-1α is highly efficient also *in vivo*: upon exposure of mice to 6% oxygen, we observed accumulation of HIF-1α protein in several organs within 60 minutes (58). This observation implies that hypoxic preconditioning for less than one hour might be sufficient to provide tissue protective effects.

EPO PROVIDES NEUROPROTECTION IN THE BRAIN

Until recently, the function of Epo was thought to be restricted to erythropoiesis. Binding of Epo to its receptor (EpoR) present on erythroid progenitor cells was shown to repress apoptosis, thereby allowing red blood cells to mature (27). However, we and others discovered expression of Epo and EpoR in the brain (13, 39, 59). The presence of the blood-brain-barrier made a systemic erythropoietic function of brain-derived Epo unlikely and suggested a local role of Epo in the brain by binding to local EpoR's. In analogy to the kidney, Epo gene expression in brain is regulated in an oxygen-dependent manner. Considering that Epo is a HIF-1-target gene, we were not surprised to find elevated Epo protein levels in the hypoxic brain (58).

Why do we need Epo in the brain? Sasaki and co-workers reported for the first time that Epo protects neurons from ischemic damage *in vivo* (51) and many subsequent animal stroke models confirmed that Epo protects against this ischemic brain injury (reviewed in (40)). By now, several reports have enlarged the possible use of Epo in ischemic-related injuries (including spinal cord) and the involved protective mechanisms have been summa-

rized recently (17, 18, 38). At the same time, Ehrenreich and co-workers (14) pioneered the first clinical trial applying rhEpo on patients suffering from acute stroke. They reported that Epo-treated patients showed markedly enhanced neurological recovery and an improved clinical outcome. The fact that no side effects of Epo therapy were identified makes the therapeutic use of Epo in ischemia-related neuronal injuries very promising.

TRANSGENIC MICE OVEREXPRESSING HUMAN EPO

Encouraged by the beneficial effects of Epo in the brain of stroke patients and animal models we started to study the potential neuroprotective capacity of Epo in the retina by three different approaches: i) by upregulation of Epo (among other factors) through hypoxic preconditioning, ii) by application of rhEpo and iii) by transgenic overexpression of hEpo. For the latter approach, a transgenic mouse line was generated that expresses human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter. This promoter directs expression of the transgene preferentially, but not exclusively, to neuronal cells. The resulting transgenic mouse line termed tg6 showed a dramatic increase of cerebral and plasma Epo levels, the latter leading to excessive erythrocytosis with hematocrit values of up to 90% (50, 55, 62). Unexpectedly, blood pressure was not elevated nor was the cardiac output altered in tg6 mice. Despite concomitant activation of the endothelin system (46), elevated NO-levels observed in tg6 mice led to a generalized vasodilation (50). In concert with regulated blood viscosity (61), the observed vasodilation protected tg6 animals from cardiovascular complications.

EPO IS OVEREXPRESSED IN THE TRANSGENIC RETINA

The transgene caused more than 20-fold elevated levels of Epo in the retina (21). The high levels of Epo did not influence retinal development, and the adult retina showed normal morphology and thickness without obvious alterations, and also a normal content and regeneration of visual pigments (21). However, the retinal vessels were massively dilated, in particular the veins (Fig. 1A). The functional analysis was performed to answer the question whether enhanced hematocrit would lead to a reduction (e.g. via a reduced capillar flow) or an enhancement of the electrophysiological activity. It turned out that retinal function was enhanced in tg6 mutant mice. Both rod and cone system function as measured by the scotopic (Fig. 1B) and photopic (not shown) electroretinogram (ERG) were increased in comparison to normal, especially at higher stimulus intensities (Fig. 1B). The exact reason for the enlarged ERGs is not clear but presumably involves alterations in the blood and oxygen supply to the retina and/or the retinal pigment epithelium (RPE), caused by the high hematocrit values, vasodilation and altered blood viscosity in tg6 mice. Alternatively, subtle changes in retinal architecture, especially in the inner nuclear layer (INL) and Muller cells may contribute to the increased b-wave amplitudes. Such potential structural changes in the tg6 retina may not be detectable in histological sections examined by conventional light microscopy. A more detailed analysis of the tg6 retina with respect to the observed functional alterations is currently in progress. Although these experiments are not yet completed, we used the tg6 mouse to test the potential benefit of increased Epo

levels on the survival of retinal cells in mouse models of induced and inherited retinal degeneration.

Light is considered a risk- or cofactor for many human retinal degenerations (10-12, 57, 60). Furthermore, degeneration in many animal models is accelerated by light and light-induced retinal degeneration in wildtype mice has been used to study pathophysiological mechanisms in the retina (8, 9, 33, 44, 52, 63, 66). Short exposure of mice to high levels of white light induces a synchronized burst of apoptosis in a large number of photoreceptors. This process leads to cell death, loss of retinal function and blindness (47, 48, 66). Since apoptosis is the final common death pathway in most human retinal diseases, this system can be used to study signaling pathways and apoptotic cascades in the retina with respect to human pathology.

Many transgenic and spontaneously arised mouse lines exist that represent models for inherited retinal degenerations (22). Results obtained in the induced system can be tested in some of these models especially to analyze effectivity of therapeutical approaches like gene therapy, implantation of prosthetic devices and neuroprotective treatments (see below).

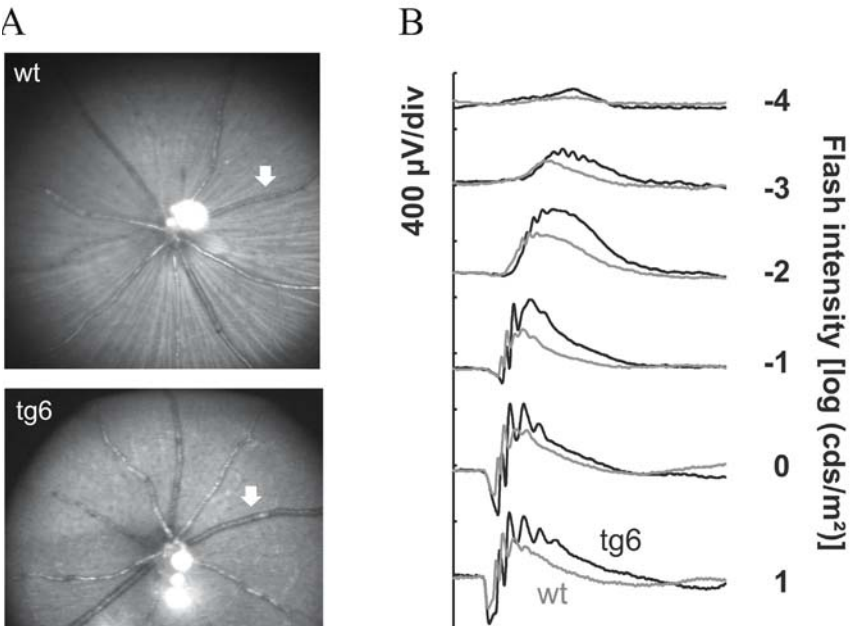


Figure 1. Functional and morphological *in vivo* analysis of tg6 mutant mice (see Ref (26) for methodological details on the scanning laser ophthalmoscope (SLO) and ERG analysis). A) Analysis of retinal vessels *in vivo* by SLO reveals dilatated retinal vessels in the tg6 mouse. The comparison between wt and tg6 mice shows that particularly the veins have a massive increase in diameter (arrows). B) Enlargement of ERG amplitudes in tg6 mice. Both the negative (a-wave) and positive (b-wave) portion of the signals is increased.

WHY THE RETINA?

The retina is a highly specialized and easily accessible 'outpost' of the brain designed to convert light into an electrical signal that can be interpreted by the brain. This demanding task is carried out by the different cells of the retina with photoreceptors being the light-absorbing cells. The extreme specialization of these cells renders them highly vulnerable and their physical and physiological integrity is easily disturbed by environmental factors as well as by gene mutations.

Many human blinding diseases exist in which cells of the retina, in particular photoreceptors, are lost through an apoptotic cell death. As a consequence, the retina degenerates leading to partial or complete loss of vision. In the gene for the visual pigment rhodopsin, over a hundred mutations are known that lead to Retinitis Pigmentosa (RP). To date, no effective treatment exists for most degenerative diseases of the retina. Strategies to develop therapies include retinal prosthesis, stem cell transplantation, gene therapy, surgery and anti-apoptotic or neuroprotective measures to interfere with the cell death program. To inhibit cell death in various models of retinal degeneration, several anti-apoptotic factors have been used with different success. Tested factors included members of the Bcl-2 family of proteins, growth factors and cytokines (66). Similarly, several protocols of preconditioning, including hyperthermia, food deprivation, light exposure or injury of the retina were shown to protect the retina against degeneration (1, 5, 30, 35-37, 41-43, 67). Likewise, ischemic preconditioning conferred protection against apoptotic stimuli like ischemia-induced death of ganglion cells and light-induced photoreceptor degeneration (6, 37, 69).

HIF-1α IS UPREGULATED DURING POSTNATAL DEVELOPMENT OF THE RETINA

The mouse retina fully develops during the first three weeks after birth. This postnatal development is characterized by the final differentiation of the various retinal cell types, the expression of the visual pigment and the development of the retinal vasculature. Until retinal vessels have fully developed, the retina may experience reduced oxygen concentrations. Using RT-PCR, we show that during this critical period of retinal development as well as in the adult mouse retina, both HIF-1α and HIF-1β/ARNT are expressed at constantly high levels (Fig. 2A). HIF-1α protein, however, can be detected at high levels only during early postnatal development (Fig. 2B). Concomitant with the development of the retinal vessels of the inner retina, HIF-1α protein levels decrease, presumably because the retina becomes more and more oxygenized. High levels of HIF-1 transcription factor during postnatal retinal development most likely secures proper retinal vasculogenesis by regulating expression of proteins involved in angiogenesis like VEGF. In the adult retina, HIF-1α is barely detectable. Nevertheless, the genes for both HIF-1 subunits are still highly expressed suggesting that the retina may be able to quickly react to any reduction in oxygen tension. Stabilization of HIF-1α with subsequent differential regulation of HIF-1 target genes in reduced oxygen conditions may be an important neuroprotective mechanism in the adult retina. This may particularly be necessary during nighttime, when the retina has the highest energy demand and oxygen conditions are borderline hypoxic in the retina.

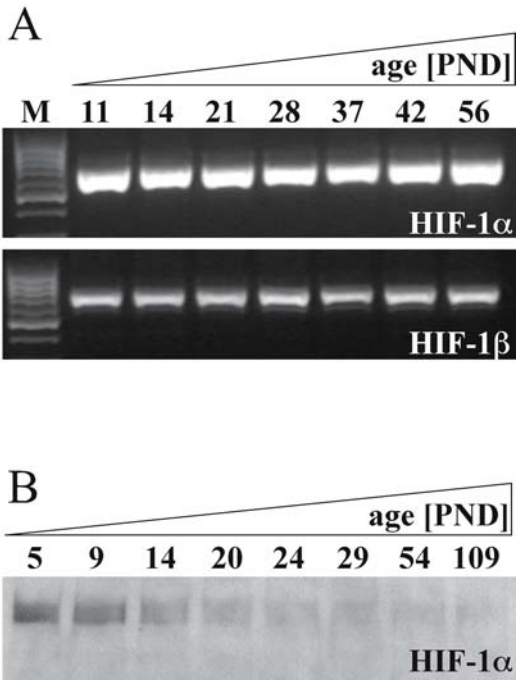


Figure 2. HIF-1α and HIF-1β expression during retinal development. A) Total RNA was prepared from isolated retinas at different post-natal days (PND) as indicated. RNA was reverse transcribed and amplified using specific primers for HIF-1α and HIF-1β, respectively. B) Total protein was prepared from isolated retinas at different post-natal days as indicated. HIF-1α levels were tested by Western blotting using a specific antibody (4).

NEUROPROTECTION IN THE RETINA THROUGH HYPOXIC PRECONDITIONING AND HIF-1 ACTIVATION.

We intended to prepare the adult mouse retina to cope with a strong apoptotic stimulus by a short exposure to low environmental oxygen concentrations. We then analyzed the molecular response of the retina to this hypoxic preconditioning and tested the neuroprotective capacity of this treatment (20). Hypoxic preconditioning stabilized HIF-1α in the retina and levels of retinal HIF-1α protein followed a dose-response function: exposure to 6% and 10% oxygen for 6 hours strongly stabilized HIF-1α, while exposure to 14% oxygen had an intermediate effect on HIF-1α levels, and 18% and 21% (normoxia) conditions did not cause any notable stabilization of the transcription factor. As noticed in other tissues, HIF-1α was quickly destabilized upon reoxygenation and protein levels reached basal values already after 1 hour in normal room air (20). The functionality of the stabilized HIF-1α protein was shown by the increased expression of HIF-1 target genes in the retina. Of particular interest was the strong induction of erythropoietin (up to 10-fold) (20).

Hypoxic preconditioning induced a neuroprotective response in the retina rendering photoreceptors resistant against exposure to high levels of white light, a strong apoptotic insult (48, 66). Without preconditioning, exposure to 5'800 lux of white fluorescent light for one hour was sufficient to induce apoptosis in a large number of photoreceptors leading to cell loss and retinal degeneration. Photoreceptors of mice preconditioned by hypoxic exposure, however, were completely protected against the light insult when exposed after

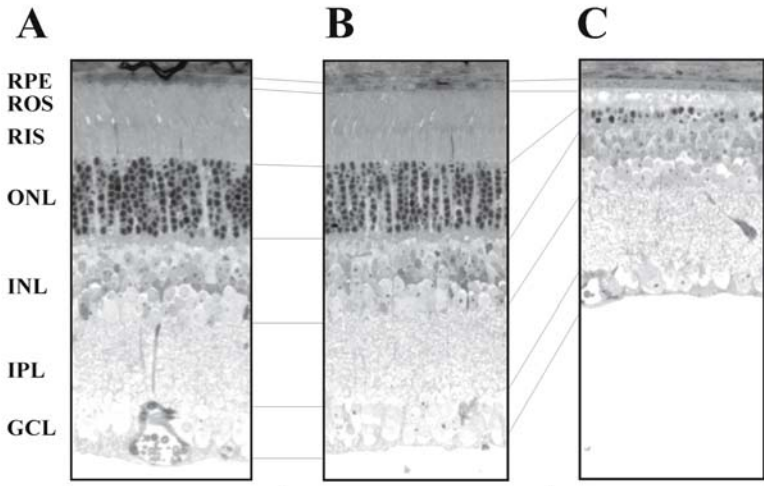
a 4-hour period of reoxygenation (Fig. 3A). Importantly, not only retinal morphology was protected but also retinal function as determined by ERG recordings (20). After 16 hours of reoxygenation, photoreceptors were again susceptible to light damage and light exposure resulted in photoreceptor loss and thinning of the outer nuclear layer (ONL) as well as of the layer of rod inner (RIS) and rod outer segments (ROS). All other layers including the RPE remained remarkably intact (Fig. 3B). The transient nature of the neuroprotective effect indicates that that the protective factors induced by hypoxic preconditioning are short-lived and implies that these factors might be deactivated immediately upon reoxygenation to restore a physiological retinal environment. Neuroprotection induced by hypoxic preconditioning most likely was dependent on the transcription factor HIF-1 since protection correlated directly with retinal levels of HIF-1α and inversely with oxygen concentrations during the preconditioning period (20).

Other potential protective mechanisms involve stress-mediated protection (67) or severely affected rhodopsin regeneration in the visual cycle (31, 56, 68). Light damage as applied here utilizes signaling cascades involving the activation of the transcription factor AP-1 (23, 64, 67). Stress was shown to inhibit AP-1 DNA binding via the activation of the glucocorticoid receptor (GR) in the retina (67). Although hypoxic preconditioning indeed imposed a stress on the animals leading to an activation and nuclear translocation of the glucocorticoid receptor (GR) immediately at the end of the hypoxic period, nucleo-cytoplasmic distribution of GR was normal and indistinguishable form normoxic controls after the 4-hour period of reoxygenation that preceded light exposure (20). Furthermore, light exposure of hypoxic preconditioned mice strongly activated AP-1 activity without leading to photoreceptor apoptosis. This suggests that protection was not mediated through the inhibition of AP-1 and that hypoxic preconditioning interrupts the apoptotic signaling cascade downstream of AP-1. Another important parameter for light induced retinal degeneration is the speed of the visual cycle. Upon photon absorption 11-cis retinal is photoisomerized to all-trans retinal in the visual pigment rhodopsin. Before rhodopsin can absorb another photon, 11-cis retinal has to be regenerated from all-trans retinal in the visual cycle, a complex reaction cascade involving a multitude of enzymes in both the RPE and the photoreceptors. Slowing or blocking the visual cycle renders the retina resistant against light damage due to the reduced number of photon absorptions by rhodopsin (31, 56, 65, 68). It has been shown that hypoxic exposure alters rhodopsin regeneration in mice (45) as well as dark adaptation in humans (2, 32, 34). However, after 4 hours of reoxygenation – at the timepoint of light exposure – rhodopsin regeneration was comparable in preconditioned and control mice (20). Neuroprotection against light toxicity might therefore not be mediated by alterations in the visual cycle but rather by factors differentially regulated by the hypoxic period. Such factors may not be stably expressed under normoxic conditions and their production might depend on oxygen-sensing molecules like HIF-1.

EPO AS NEUROPROTECTIVE AGENT IN THE RETINA

One of the most prominent genes induced by hypoxia via HIF-1 is Epo. Using real-time PCR, we showed that Epo was strongly upregulated in the retina upon hypoxic exposure (20). In addition, Epo receptor was found to be expressed on photoreceptor cells (20) and retinal ganglion cells (29). Epo protects neuronal cells from apoptotic cell death in a variety

of models for neuronal damage (15) including the retina (29). We therefore tested whether Epo is responsible for the retinal protection observed after hypoxic preconditioning. Recombinant human Epo (rhEpo) was injected intraperitoneally into normal mice before or after light exposure. This treatment reduced light damage susceptibility of the mice, suggesting that Epo-mediated activation of the Epo receptor directly induces protection of the visual cells. However, protection by rhEpo was less complete than the protection observed after hypoxic preconditioning. It is possible that the limited protective capacity of rhEpo was due to a poor translocation of the protein across the blood-retina barrier leading to an insufficient availability of Epo in the retina. However, even the 20-fold increased retinal Epo levels in tg6 mice did not induce the same complete protection as observed after hypoxic preconditioning (21). This result suggests that pretreatment with low oxygen induces several factor(s) (Epo being one of them) that may need to act in concert to fully protect photoreceptors against a light-induced injury.



Prec	6h	6h	6h
Reox	4h	4h	16h
Light	No	Yes	Yes

Figure 3. Hypoxic preconditioning induces a transient neuroprotection of the retina against light-induced photoreceptor apoptosis. All BALB/c mice were preconditioned (Prec) for 6h with 6% oxygen, reoxygenized (Reox) for 4 hours (A, B) or 16 hours (C) prior to exposure to 1 hour of 5'800 lux of white light (Light; B, C). Retinal morphology was assayed on plastic embedded sections by light microscopy 10 days after light exposure. A) Retinal morphology of a control mouse without light exposure. B) Retinal morphology of a light exposed mouse preconditioned with hypoxia followed by a 4-hour reoxygenation period. The retina was completely protected against light induced apoptosis. C) Retinal morphology of a light exposed mouse preconditioned with hypoxia followed by a 16-hour reoxygenation period. The retina was susceptible to light induced apoptosis. Retinal degeneration and loss of photoreceptor cells is indicated by the thinning of the outer nuclear layer (ONL), rod inner segments (RIS) and rod outer segments (ROS). The other layers of the retina were not affected by the exposure to light. Shown are representative sections of at least 4 different animals. RPE: retinal pigment epithelium, INL: inner nuclear layer, IPL; inner plexiform layer, GCL: ganglion cell layer.

EPO IN MODELS OF INHERITED DEGENERATION OF PHOTORECEPTORS

Many mouse models of inherited retinal degeneration exist (22). To test the therapeutic potential of Epo, we used two models: the retinal degeneration mouse 1 (rd1) as a model for autosomal recessive Retinitis Pigmentosa (RP) and the VPP transgenic mouse resembling an autosomal dominant form of RP. The rd1 mouse carries a spontaneous insertion of a retrovirus in the gene encoding the β-subunit of phosphodiesterase leading to aberrant splicing and a null phenotype (7, 66). The lack of phosphodiesterase activity increases levels of cGMP in photoreceptors resulting in constantly open ion channels and, as a consequence, elevated intracellular Ca^{2+} levels. Either the elevated cGMP and/or the high levels of Ca^{2+} might be the cause of the observed early onset (around post natal day 10) and rapid degeneration of the photoreceptor cell layer. Already ten days after onset of degeneration, retinas of rd1 mice are mostly devoid of rods.

The VPP mouse expresses a transgene encoding a mutant form of rod opsin with three amino acid substitutions at the N-terminal end of the protein (19). One of these mutations causes the substitution of prolin at residue 23 by histidine (P23H). This mutation is the most frequent mutation found in opsin of human RP patients and accounts for about 10% of autosomal dominant RP in the USA.

Two approaches were used to investigate the potential neuroprotective capacity of Epo in inherited retinal degeneration: i) recombinant human Epo (rhEpo) was systemically applied by intraperitoneal (ip) injections into rd1 and VPP mice every other day before onset and during the first phase of degeneration. ii) using classical breeding schemes, both mouse models for inherited retinal degeneration were combined with the transgene in tg6 that expresses Epo at high levels especially in neuronal tissue including the retina (see above). However, neither the transgene nor the systemic application of rhEpo protected the rd1 or the VPP mouse against retinal degeneration (21). Protection of photoreceptors in the rd1 and the VPP mouse may therefore require other or additional factors. We are currently attempting to identify the postulated (see above) additional neuroprotective molecules induced by hypoxic preconditioning. Once identified, it will be important to test these factors in the inherited models. Recently, it has been suggested that only systemically but not locally produced Epo is neuroprotective in the retina (49). Since the tg6 mice seem to produce most of their Epo locally in neuronal tissue, this observation might additionally explain the weaker neuroprotective capacity – as compared to hypoxic preconditioning – of the transgene against light toxicity. Additional experiments have to be conducted in order to establish the physiological and neuroprotective role of Epo produced locally in the retina.

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Chapter 12

BLOCKING STRESS SIGNALING PATHWAYS WITH CELL PERMEABLE PEPTIDES

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Abstract: Cells are continuously adapting to changes in their environment by activating extracellular stimuli-dependent signal transduction cascades. These cascades, or signaling pathways, culminate both in changes in genes expression and in the functional regulation of pre-existing proteins. The Mitogen-Activated Protein Kinases (MAPKs) constitute a structurally related class of signaling proteins whose distinctive feature is their ability to directly phosphorylate, and thereby modulate, the activity of the transcription factors that are targets of the initial stimuli. The specificity of activation of MAPK signaling modules is determined, at least for an important part, by the specificity of the protein-protein contacts that are required for the propagation of the signal. We will discuss how we may interfere with MAPK signaling by using short cell-permeable peptides able to block, through a competitive mechanisms, relevant protein-protein contacts, and their effects on signaling and cell function.

Key Words: signaling, apoptosis, cell-permeable peptides, MAPK

INTRODUCTION

Extracellular stimuli activate different signal transduction pathways that culminate in modification of pre-existing proteins, changes in mitochondrial permeability, ER responses and changes in gene expression, ultimately, robust and durable stimuli may lead to apoptosis. Amongst other signaling components, MAPK (Mitogen-Activated Protein Kinases) constitute the final elements of a series of signaling events that transduce external stressful events to the nucleus, the mitochondria, the ER, and other cell components. The distinctive feature of MAPKs is their ability to directly phosphorylate, and thereby modulate the activity of the transcription factors that are targets of the initial stimuli.

Regulation and activation of MAPKs appears to require sophisticated signaling events,

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thus providing different therapeutic opportunities. MAPK core module pathways are com posed of three conserved kinases that proceed through a phosphorylation cascade creating a sequential activation pathway. The first kinase in the module is a MAPK kinase kinase (MKKK), a serine/threonine kinase that when activated phosphorylates and activates the next kinase in the module, a MAPK kinase (MKK). The MKKs are dual specific kinases that phosphorylate a Thr-X-Tyr motif in the activation loop of MAPKs. MAPKs are the final kinases in the module and phosphorylate different substrates on serine and threonine residues. Three major conserved groups of MAPKs have been described: the Extra-cellular signal Regulated Kinases (ERK1/2/3) (10), the p38 kinases (p38 α/ß/γ/and θ) (27), and the c-Jun NH_2-terminal kinases (JNK1/2/3) (13).

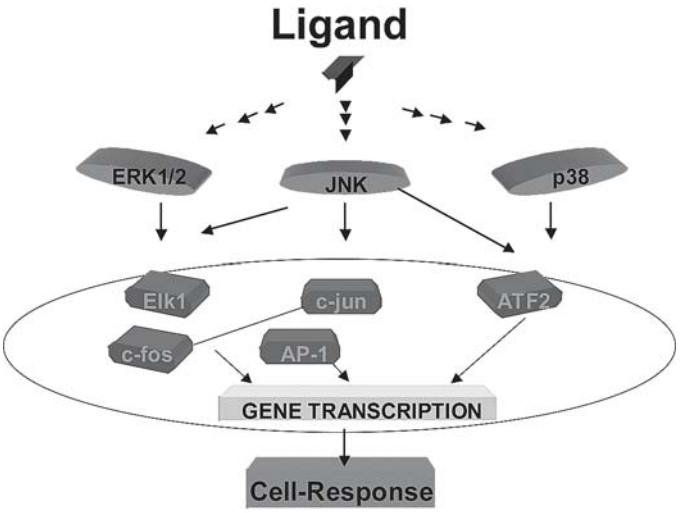

Figure 1. MAPK signaling unit is made up of three sequential kinases. The signal is transmitted to the nucleus through three MAPKs, ERK, p38 and JNK.

In mammals, these MAPKs can be activated by six MKKs: MKK1 and MKK2 are upstream activators of ERK1/2, MKK3 and MKK6 are activators of p38, and MKK4 and MKK7 regulate JNK. It has also been shown that MKK4 can activate p38 in addition to JNK. Both JNK and p38 activate downstream nuclear transcription factors that participate to the cellular response. Amongst these, activation of the activator protein-1 (AP-1) ?formed of heteromers of c-Fos, c-Jun and ATF2- is required for some forms of apoptosis, notably in neuronal cells. A major transcriptional target of JNK and p38 is the c-fos gene. The p38 kinases and JNKs primarily respond to a variety of stimuli collectively designated as «stress-signals». These include for example cytokine treatments (IL-1ß, TNFα), but also environmental stresses including osmotic shocks, heat and cold shocks, lack of survival factors, uncoupling from the cell-matrix, shearing stresses, reactive oxygen species (12, 37) and many others.

JNK

10 different isoforms of JNK arising from the expression of three genes have been described. Amongst these, the JNK3 isoforms appear particularly interesting as they show expression restricted to neurons and heart, and they have been clearly associated with apoptotic events in the brain, potentially through the induction of Bim and Fas and the mitochondrial release of cytochrome c (30, 48), JNK3 KO mice are substantially resistant against and survive kainic acid-induced seizures (11). Furthermore, the JNK1, 2 and 3 isoforms show clear substrate specificities (21), explaining at least in part why only the JNK3 protein has been associated with cell-death in some neuronal populations. Excluding transcription factors, JNK also phosphorylates a whole battery of cytoplasmic, membrane-bound and mitochondrial targets. These include: bcl-2, bcl-xL, MADD or IRS-1.

P38

The family of the p38 proteins regroups 4 major isoforms: α/ß/γ/θ. The p38α and ß isoforms, which are 75% homologous, are ubiquitously expressed and have been intensively studied, as numerous inhibitors are readily available, the most widely used being the SB203580 and SB202190 agents (38). It is important to note here that these inhibitors do not affect the γ and θ isoforms, although they strongly inhibit a whole battery of other kinases, rendering the interpretation of data generated using these chemicals extremely difficult (the same situation being true as well for the commercially available JNK inhibitors) (4). No chemical inhibitor is available to block neither the p38γ nor the θ isoforms. The expression of the p38γ form is limited to muscles, whereas p38θ shows preferential expression in the pancreas. All p38 isoforms are activated by the same upstream kinases ? MAP kinase kinase (MKK)3 or MKK6 ? in response to inflammatory and stressful stimuli. A major substrate of p38α and ß is MAPK-activated protein kinase (MAPKAPK)-2. The p38γ and θ forms are less effective at activating the downstream kinases and better at phosphorylating transcription factors such as activating-transcription-factor (ATF)2, Elk-1 and SAP-1 (38). As we will show, there is clear overlapping between the substrate specificities of p38θ and JNK, both being for example able to phosphorylate the N-terminal transcriptional activation domain of c-Jun. Finally, the activity of p38θ is regulated by the scaffold protein IB2 (35, 40), the second member of the Islet-Brain (IB) family of proteins, a genetic defect in IB1, which regulates JNK signaling, has been linked to diabetes (47).

Ample evidence suggests a correlation between the activation of the p38 pathway and apoptosis. Such a correlation is based on the concomitant activation of p38, caspase activation and apoptosis induced by a variety of agents including NGF withdrawal and Fas ligation (this being true for JNK as well). Overexpression of dominant active MKK6ß can induce caspase activity and cell death (16), the mechanism by which this pathway might influence caspase activity being currently not fully characterized. Also, SB203580 can block sodium salicylate-induced FS-4 fibroblast apoptosis, glutamate-induced cerebellar granule cell apoptosis, serum depletion induced Rat-1 cell death, NGF withdrawal-induced PC12 cell apoptosis, and TL1-induced bovine pulmonary artery endothelial cell apoptosis (see (36) for a review on the p38 signal transduction pathway). Activation of p38θ has been

shown to be required for anoikis of enterocytes induced by inhibition of focal adhesion kinase or beta1 integrins, or by maintaining cells in suspension (46). The respective roles of the p38ß and p38θ isoforms might be clearly antagonist, as for example the former isoform increases LPS-dependent induction of Heme Oxygenase (HO)-1 gene expression, whereas activation of the latter prevents its expression (54). In keratinocytes, activation of p38θ is associated with apoptosis (16). In these cells, okadaic acid, which is a strong activator of p38θ but not the other p38 isoforms, as well as overexpression of p38θ inhibits ERK1/2 activity without reducing ERK1/2 levels. In keratinocytes again, p38θ activity is associated with increased levels of AP1 binding protein factors. This response is maintained in the presence of SB203580, whereas dominant-negative p38θ inhibits AP-1 activity (15). Thus, p38θ may directly suppress ERK1/2 activity at the same time as activating AP-1.

EVIDENCE THAT JNK AND P38 SIGNALING IS ORDERED BY SPECIALIZED SCAFFOLD PROTEINS

Many intracellular components that participate to signaling events are presumably physically ordered through distinct protein-protein interactions. The specificity of activation and function of MAP-kinase signaling modules appears determined, in part, by scaffold proteins that create multienzyme complexes. These scaffold proteins facilitate MAP-kinase activation in response to specific physiological stimuli, and insulate the bound MAP-kinase module against activation by irrelevant stimuli. Scaffold proteins are therefore critical components of MAP-kinase modules to ensure signalling specificity (14, 52).

In *Saccharomyces cerevisiae*, two MAP- kinase-scaffold proteins have been identified. In mammals, different scaffolds with no intrinsic enzymatic activity have been described that aggregate sequential components of MAP-kinases signaling. One such protein, MP1, tether the ERK kinase MEK1 to ERK (39). Two other scaffolds aggregate the JNK signaling unit: MLK-MKK7-JNK. These are JIP-1 (IB1) and JIP-3/JSAP-1, whereas JIP-2 (IB2) is a scaffold for p38θ (7, 24, 29, 35, 51, 60).

Figure 2. The IB and MP1 scaffolds aggregate sequential components of a signalling module (55, 71).

JIP-3 belongs to a structurally distinct family of proteins than the highly related JIP-1/IB1 and JIP-2/IB2. Despite their differences in structure, it is noteworthy that all these scaffolds are expressed almost exclusively in brain and also in heart (7, 29, 34, 35, 51, 60). Except for the JIP/IB proteins that are able to interact with the ApoER2 receptors and presumably with other components of LDL-receptor signaling, the known scaffolds are currently «orphan», *i.e.* no convincing association with either upstream or downstream components of the signaling cascade have been described. Results in yeast where scaffolds were known for many years and in human (47, 52), indicate that malfunction of these molecules may result in major physiopathological states (53).

USE OF CELL-PENETRATING PEPTIDES TO BLOCK SIGNALING EVENTS

Inhibiting kinase activities along intracellular pathways is usually used to block signaling. An alternative is to prevent essential protein-protein interaction between necessary factors within signaling cascades. This might be efficiently performed in cell lines, tissue or animals by cell-penetrating peptides (41) that take advantage of the often small size of the amino acids sequences mediating interaction between two protein partners. The blockade is achieved by introducing a molar excess of one of the two protein interaction domains inside cells. Importantly, this process does not require any intrinsic enzymatic activity from any of the protein partners. Intracellular delivery is achieved by linking these sequences to special peptidic transporters such as "TAT_{48-57}", "Antennapedia" and others. Probably because of its short length (10 amino acids) and its good efficacy in crossing cell-membranes from different cell-types, the TAT_{48-57} sequence from HIV seems now the most generally used for intracellular delivery of peptides. To date, different cell-penetrating peptides acting on different signaling pathways have been shown to confer biological activity in either animal or cell culture models. They include BH4 domain peptides from Bcl-xL, NEMO-Binding-Domain peptides (NBD, blocks NF-κB signaling), TRAF peptides, peptides derived from the signaling protein PSD95 (TAT-NR2B9c), cyclinD:Cdk4/6 derived peptides, hypoxia-inducible factor-1 (HIF) peptides and many others (1, 20, 33, 44, 55, 61). We have engineered similar peptides to block the interaction between JNK and its target c-Jun (6, 8), and shown their biological activity in animals (9, 49).

MAPK INHIBITORY PEPTIDES

The substrate specificity of MAPKs is determined by two components: 1? the sequence and local context of the phospho-acceptor motifs (MAPKs phosphorylate serine and threonine residues followed by proline residues) and 2˚ the sequence of the docking site on the substrate. The MAPK-docking sites locate usually outside the catalytic domain of the MAPK-interacting molecules. The first MAPK docking site or binding motif identified was the θ domain of c-Jun (42), which acts as a binding site for JNK. The θ domain is important for efficient phosphorylation of c-Jun and the regulation of its transcriptional activation.

v-Jun, the oncogenic counterpart of c-Jun, does not posses the θ domain and therefore looses the ability to bind JNK (32). While JunB, which contains a functional JNK docking site, cannot be phosphorylated by JNKs owing to the absence of sequence specificity conferred by residues surrounding its phospho-acceptors (19), insertion of specific residues can bring JunB under JNK control. JunD, by contrast, possesses a phospho-acceptor region essentially identical to that of c-Jun but lacks an effective JNK docking site, resulting in a weak phosphorylation by JNK. Nevertheless it can be phosphorylated by JNK through heterodimerization with docking competent partners (25). By using a peptide encoding the θ domain and purified JNK, it has been demonstrated that this peptide inhibits phosphorylation of the amino terminus of both c-Jun and JunD, by competing with the substrate for binding to the kinase (2).

Similar docking motifs related to the θ domain and called the docking domain or D domain, are found in MAPK-interacting proteins (18, 45, 58, 59). This docking site is typically less than 20 amino acid long and shows limited sequence similarity, but is characterized by a «slashcircle»A-X-øB motif, where øA and øB are hydrophobic residues (Leu, Val or Ile), separated by 2-6 residues from a cluster of a least two basic residues (Lys, Arg) (5, 43, 50, 56). The basic and hydrophobic residues of the D domain have been shown to be important for recognition, binding, specificity and phosphorylation. For example, the N-terminal portion of MKK4 contains a MAPK-docking site and serves as a binding site for its upstream activator, MEKK1, and its downstream target JNK. Furthermore, the N-terminal portion of MEK1 and the C-terminal portion of RSK2, both of which include the MAPK-docking site, are sufficient for binding to ERK2 (57). Further studies showed that ERK and JNK target Elk-1 via the D domain, and that this targeting is essential for efficient Elk-1 phosphorylation. However, different residues in the D domain are important for ERK and JNK binding. In contrast, p38 is not targeted to Elk-1 via the D domain. ATF2 also contains a docking domain for JNK. Deletion of the binding domain of c-Jun or ATF2 blocks their phosphorylation by JNK.

A peptide derived from the N-terminal sequence of MEK1 and containing the ERK docking domain completely inhibits the interactions between ERK and its substrates and regulators, but is much less efficient in affecting the interaction between p38 and its substrate and regulators. Experiments performed using a p38-derived peptide gave reciprocal results. Similarly, we have produced a peptide from the docking domain of IB1 that specifically prevents the interaction between JNK and its substrate, but not between p38 or ERK and their respective targets (8). By linking this domain to the HIV TAT_{48-57} transporter, we were able to produce cell-permeable molecules capable of blocking JNK signaling in vivo. Others have taken a similar approach to block either ERK (28) or p38 signaling (17).

CELL-PENETRATING PEPTIDE INHIBITORS OF JNK

As indicated above, we designed the first cell-penetrating inhibitors of JNK (JNKi peptides) by linking the minimal 20 amino acids JBD's (*J*NK *B*inding *D*omains) of IB1 or IB2 to the 10 amino acid TAT transporter (8). We showed that these inhibitors efficiently concentrated into a variety of cell types and organs in animals, prevented phosphorylation of c-Jun and ATF2 by JNK (Figures 3 & 4), and blocked the apoptotic response under different stress conditions. These molecules now represent established tools to manipulate the

JNK signaling pathway in animals (6, 8, 31). Furthermore, these JNK-inhibitory peptides have shown real therapeutic perspectives, as they were able to restore normoglycaemia in two models of type 2 diabetes, C57BL/KsJ-db/db mice and C57BL6 mice treated with a high-fat, high sucrose diet, the effect occurred mainly by improvement of insulin-resistance through blockage of JNK-mediated IRS-1 inactivation (3, 26), the same peptides also decreased brain lesions in mice and rats models of stroke (9, 23), and they conferred protection against noise- and antibiotic induced hearing loss (49).

To more precisely define the inhibitory properties of the JNKi peptides, we further analyzed 18 substrates of JNK in *in vitro* protein kinase assays, as well as the effect of the peptide on the activity of 40 different kinases. Our results indicate that the peptide does not inhibit binding of JNK to all its substrates, *i.e* some substrates, including p53 and PPARγ, are efficiently phosphorylated by JNK even in the presence of JNKi. It is not yet clear whether this partial inhibition of JNK-mediated signaling plays any role in the anti-apoptotic activity of the peptide (*i.e*., would the protective effect be less efficient if the access of JNK to *all* its substrates had been prevented?-it is possible that some of the substrates modified by JNK in presence of the peptide might have anti-apoptotic activities (22)). Apart from its action on the three JNK isoforms and the two upstream JNK-kinases MKK4 and MKK7, JNKi did not appear to significantly impair the activity of any of the other kinases tested (see also (9)).

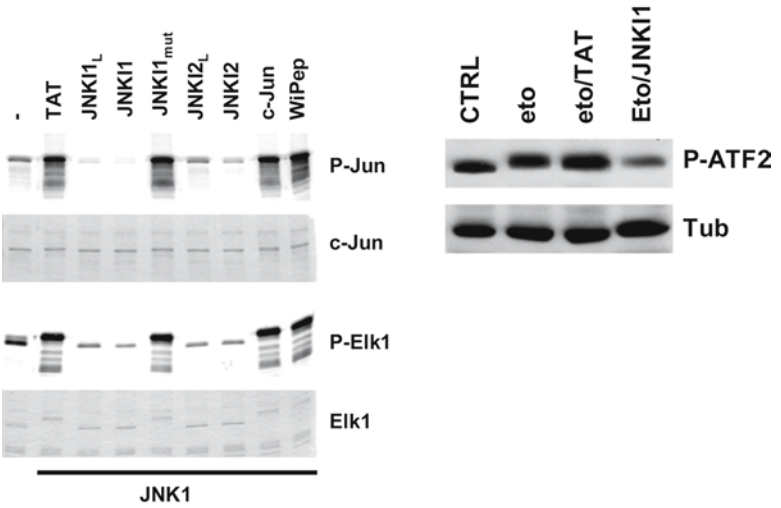

Figure 3. Left, inhibition of c-Jun and Elk1 phosphorylation by recombinant JNK *in vitro*. Right, inhibition of ATF2 phosphorylation in intact HeLa cells (eto: etoposide).

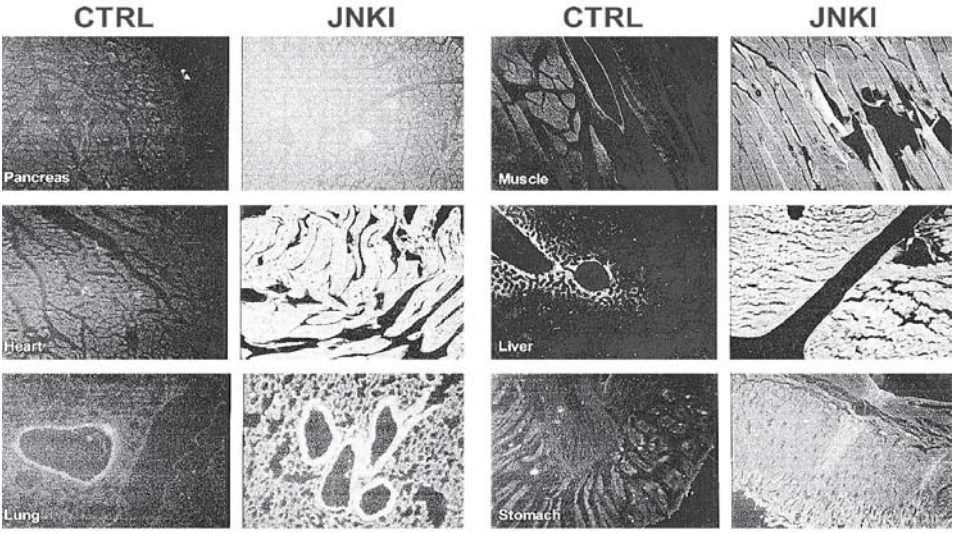

Figure 4. Penetration of JNKi in mouse tissues. 30 µl of a 1 mM solution of FITC-labelled JNKI were injected *ip* in a mouse. Tissues were mounted 1 hour later.

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Chapter 13

JNK PATHWAY AS THERAPEUTIC TARGET TO PREVENT DEGENERATION IN THE CENTRAL NERVOUS SYSTEM

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Abstract: JNKs (c-Jun N- terminal kinases) are important transducing enzymes involved in many faces of cellular regulation such as gene expression, cell proliferation and programmed cell death. The activation of JNK pathway is critical for naturally occurring neuronal death during development as well as for pathological death of adult brain following different insults. In particular, JNKs play an important role in excitotoxicity and all related phenomena. Initial research concentrated on defining the components and organization of JNK signalling cascades, but more recent studies have begun to see JNK as the appropriate target for prevent cell loss. We used a specific JNK inhibitor, the cell permeable peptide D-JNKI1, to block JNK action in neuronal death following excitotoxicity in vitro and cerebral ischemia in vivo. Here we review our recent findings and we discuss the possibility of using D-JNKI1 as a therapeutic agent to prevent cell loss in the central nervous system.

Key Words: JNK inhibitors, excitotoxicity, neurodegeneration, ischemic damage

INTRODUCTION

Excitotoxicity is defined as the excessive activation of glutamate receptors, in which *N*-methyl-D-aspartate (NMDA) receptors play a key role, owing to their high Ca^{2+} permeability. The overactivation of the NMDA receptors induces downstream cascade of events resulting in neuronal death. Excitotoxicity is responsible for many brain injuries such as ischemia (43), epilepsy (30) and it plays an important role also in several neurodegenerative diseases (42), (22).

Excitotoxicity has been studied extensively, but the downstream mechanisms coupling increased intracellular Ca^{2+} to cell death are still poorly understood. Several studies have

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tried to obtain neuroprotection by blocking NMDA receptors, the major agent representing this category is the NMDA-r antagonist MK-801: notwithstanding the promising results in animal models (reduction of the excitotoxic damage), they had to be abandoned for the emergence of unacceptable psychotomimetic side effects. These side effects are strongly correlated with the physiological NMDA receptor activity, which is essential for normal neuronal cell functions. A second possible approach in the prevention of excitotoxicity is to target specific intracellular pathways, which normally act downstream from the glutamate receptor, without blocking synaptic activity. A peptide that disrupts the interaction of NMDArs with the postsynaptic density protein PSD-95 has been used against excitotoxicity in cultured neurons and ischemic damage in vivo (1).

The first evidence of the JNK involvement in excitotoxicity derived from the analysis of JNK3 deficient mice: after a systemic injection of kainate these mice showed a reduction in seizures activity and a prevention of apoptosis (39). Moreover, mice with an inactive form of c-jun (Jun AA, in which serines 63 and 73 are mutated to alanines) also showed resistance to excitotoxic neuronal death, thus suggesting that preventing JNK from accessing c-jun might confer neuroprotection (3). It has been shown that in cortical neurons NMDA mediated neurotoxicity activates JNK in dependence on entry of extracellular calcium (23). Moreover in an immortalized rat hippocampal neuronal cell line, the post-synaptic density protein 95 (PSD95) links GluR6-mediated excitotoxicity with JNK via MLK2/3 (29). A recent paper by Brecht (11), has proved distinct roles for the different JNK isoforms in four models of neurodegeneration in vivo, including permanent ischemia and excitotoxicity by application of kainic acid.

JNK INHIBITORS

Several research laboratories have proposed JNK inhibitors in order to find effective drugs for the treatment of a variety of pathologies. These inhibitors could be divided in two classes: chemical compounds and cell permeable peptide inhibitors.

Chemical compounds

This approach consists in producing small organic compounds that are competitive inhibitors of the ATP-binding site of the kinase. This technique has given a couple of efficient JNK inhibitors. Two of them, CEP-1347 and SP600125 have been the subjects of peer-reviewed studies as described in Bozyczko-Coyne and Bogoyevitch reviews (10), (5) but the published information on their efficacy in clinical trials remains limited.

CEP-1347 is a semi-synthetic inhibitor of the MLK group of MAPKKK (see Figure 1). It blocks the upstream component in the JNK pathway, and derives from the naturally occurring small molecule K252a. It competes with ATP to bind MLKs, with IC_{50} values of 38nM (MLK1), 51nM (MLK2), and 23nM (MLK3) (26). CEP-1347 is able to prevent neuronal cell death in vivo models of Alzheimer's disease (AD), Parkinson's disease (PD) and cochlear hair cell death (37, 38).

SP600125, developed by Celgene, is an ATP competitive inhibitor of JNK (see Figure 1). It inhibits all three JNK isoforms, with IC_{50} values of 40, 40, and 90nM for JNK1, JNK2 and JNK3 respectively. It also has an inhibitory activity against other MAPKs, such as ERK and p38 (4). In vivo this compound inhibited leukocyte recruitment in a rat inflamed

lung model (16) and was active in the rat rheumatoid arthritis model (18).

It also protected dopaminergic neurons in the MPTP model of Parkinson's disease (38) and it was used to attenuate apoptosis in a model of Alzheimer's disease neurotoxicity (25).

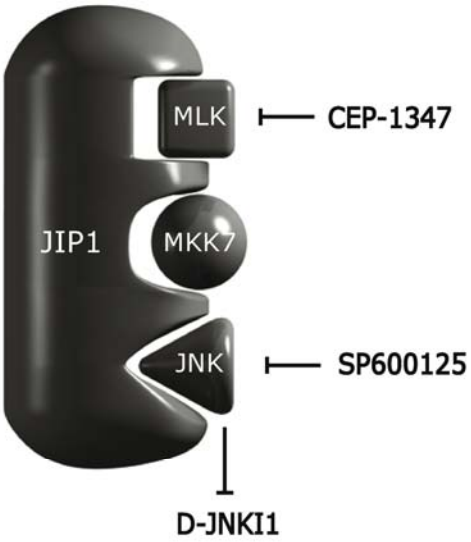

Figure 1. Modular organization of the JNK signaling pathway. JIP-1 scaffold protein binds to JNK, MKK7, one of the two JNK activators, and to members of the mixed-lineage protein kinases (MLK) group. The assembly of the JNK module by a scaffold may lead to a more efficient activation of JNK. The two small chemical inhibitors of the JNK pathway are acting at different levels of the cascade: whilst CEP-1347 inhibits MLK, SP600125 inhibits JNK activation. The cell permeable peptide D-JNKI-1 prevents JNK action without interfere with JNK activation/ phosphorylation.

Cell permeable peptide inhibitor

D-JNKI-1 inhibitor produces an allosteric modulation of JNK by blocking the access of the kinase to some of its targets, preventing protein-protein interactions, without interfering with its activation (Figure 1).

In 2001 Bonny showed that a new efficient and specific inhibitor of JNK action was able to protect pancreatic ß-cells from IL1 ß induced apoptosis. D-JNKI-1 peptide (which is the D-retro-inverso form, made of aminoacids in reversed sequence order) has been engineered by linking the 20 amino acid JNK-inhibitory sequence of IB1/JIP-1 scaffold protein (JBD_{20}) to the 10 amino acid HIV- transporter sequence (6). The TAT_{48-57} peptide penetrates in a variety of cells and could be useful for delivering macromolecules, also to animal tissues (32). Between the TAT sequence and the JBD_{20} sequence two proline-residues were inserted as spacer to allow for maximal flexibility. JIP-1 scaffold protein and c-Jun, JNK main target, share a similar binding motif for interact with JNK. However JNK binding affinity to JIP-1 is about 100-fold higher than to c-Jun, for this reason the inhibitor peptide is able to block the access of JNK to its substrates. Barr and collaborators (2) found that a shorter peptide sequence (RPKRPTTLNLF=TI-JIP) based on amino acids 143-153 on the $JBD_{20}$of JIP-1, partly overlapped with the sequence described by Bonny (6) and was also able to prevent c-Jun phosphorylation. Thus the inhibitory action of D-JNKI-1 peptide is fundamentally different from that of classical small chemical inhibitors (7), (8). D-JNKI-1 does not inhibit JNK's enzymatic activity, but selectively blocks access to many of its substrates by a competitive mechanism

(6), (7), (8). To determine the specificity of the peptide in blocking JNK action, we characterized the effects of the peptide on the activity of 40 different kinases (10 μM peptide, 10 μM ATP) towards their respective substrates in cell free system. It did not interfere with the activities of the other kinases (7), (8), proving its exceptional superiority.

In a recent study we showed that the D-JNKI-1 peptide completely inhibited excitotoxicity in primary culture and strongly prevented neuronal loss against two different models, transient and permanent, of middle cerebral artery occlusion (MCAo) (8). However other authors have demonstrated powerful protection in different models of CNS injury: D-JNKI-1 protects against toxic drug and acoustic trauma induced auditory hair cell death (36) and against retinal ganglion cells death following optic nerve crush (34). This cell permeable peptide inhibitor offers interesting possibilities for therapeutic application in preventing neuronal loss.

OUR OWN RESULTS BY USING D-JNKI-1

We investigated the protective D-JNKI-1 action at three different levels: i) in vitro in cortical neurons cultures, ii) in organotypic hippocampal cultures, and iii) in vivo in focal cerebral ischemia.

Cortical neurons

D-JNKI-1 completely inhibited cell death induced by excitotoxicity (100μM NMDA) in primary cortical neurons (Figure 2)(8). JNK activation in NMDA-treated neurons appeared maximal after 30 min of NMDA, and resulted into an elevated c-Jun phosphorylation. Addition of D-JNKI-1 completely prevented the increase in phosphorylated c-Jun after 5h exposure to 100 μM NMDA (despite a normal level of JNK activation), bringing the level of P-c-Jun below even that in the control. NMDA-induced activation of the c-fos gene, transcription of which is under the positive control of the JNK target Elk-1, was also completely prevented by the peptide. In fact, c-fos expression in D-JNKI-1/NMDA treated neurons, evaluated by real-time RT-PCR, was comparable to control levels whereas in neurons treated with only NMDA it increased and strongly correlated with the NMDA time course.

Organotypic cultures

NMDA treatment (100 μM) in organotypic hippocampal cultures (9) resulted in death of pyramidal neurons in the CA1 and CA3 already detectable within two hours of NMDA administration. In the same regions, following two hours of NMDA, c-Jun was selectively phosphorylated and c-fos was up-regulated. Pretreatment of 2 μM D-JNKI-1 prevented pyramidal neurons cell death and completely inhibited c-Jun phosphorylation and c-fos expression (Figure 3).

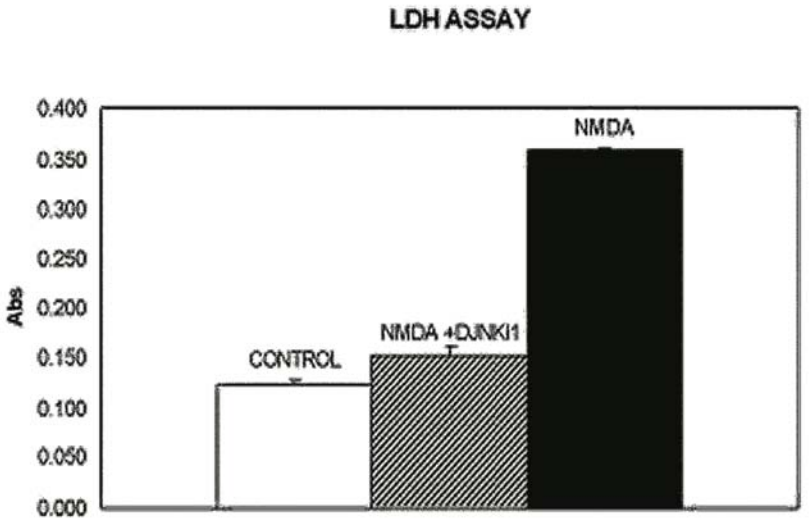

Figure 2. Neuronal death at 5 h after exposure to 100 μM NMDA evaluated by LDH activity in the culture medium in presence (black and white) or absence (black) of D-JNKI1 2 μM.

In vivo

D-JNKI-1 effect has been tested on two different models of cerebral ischemia (8), transient ischemia in adult mice and permanent focal ischemia in P14 rats. The peptide was able to confer strong protection against ischemic damage in both models. The infarcted volumes were traced on serial sections by using the Neurolucida software program after 24h from insult (17), allowing the quantification of brain damage (Figure 4). In the transient ischemia model we obtained a reduction of the infarct of 88% following an intracerebroventricular injection 1 hour before the lesion, and the strongest reduction (93%) following D-JNKI-1 administration 6 hours after ischemia. In the permanent middle cerebral artery occlusion (MCAo) model we injected the peptide intraperitoneally 30 min before, 6 hours after or 12 hours after ischemia and we obtained an infarcted volume decrease of respectively 68%, 78% and 49%. Actually, the only approved therapy for ischemic stroke is the administration of tissue plasminogen activator (tPA) within 3 hours of the onset of the ischemia, since after 3 hours the risk of a hemorrhage caused by tPA becomes too high. The most prominent feature of the protection observed with D-JNKI-1 is that the peptide is still effective when administered 6-12 hours after the onset of stroke. This is particularly relevant, as most patients will access medical centres only 6-10 hours following the onset of stroke.

To assess whether the peptide really prevented neuronal death or only delayed it, we analyzed lesion volumes one week (permanent ischemia) or two weeks (transient ischemia) after lesion in animals treated with D-JNKI-1 6 hours following ischemia, compared to control animals. In permanent ischemia D-JNKI-1-treated rats and control rats presented a shrunken infarct, but there was still a significant protection by the peptide (54%). In transient ischemia of adult mice we obtained 93% reduction of lesion volume, exactly the same

protection shown at the shorter survival time (24h after ischemia).

Furthermore, the powerful neuroprotection obtained is accompanied by behavioral benefits; we have not so far detected negative side effects at the dose-levels required for protection.

Therefore, JNK inhibition using D-JNKI-1 might prove useful in the treatment of stroke damage.

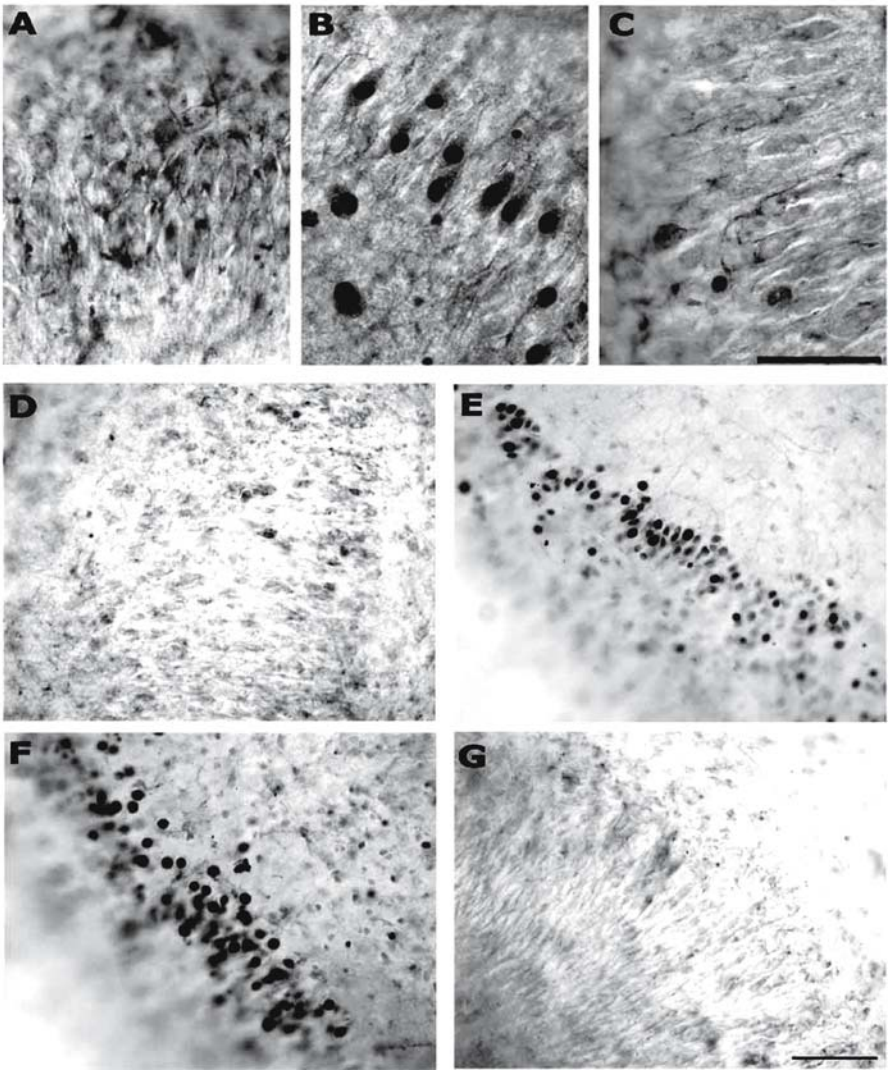

Figure 3. Immunohistochemistry in organotypic hippocampal cultures. (A-C):phosphorylated c-jun immunolabelling. (A): control condition, (B): 2h of 100µM NMDA, (C): 2h of 100µM NMDA in presence of 2µM D-JNKI1. (D-G): c-fos immunolabelling. (D): control condition, (E):1h of 100µM NMDA, (F): 2h of 100µM NMDA, (G): 2h of 100µM NMDA in presence of 2µM D-JNKI1. Reprinted, with permission from the Eur. J. Neurosci. 2003, 18(3): 473-85.

JNK TARGETS RELATED TO NEURODEGENERATION

JNK substrates related to cell death can be nuclear or cytoplasmic, and this means that JNK could act at transcriptional or at post-transductional level.

Concerning nuclear substrates, JNKs activate several members of the AP-1 group of transcription factors, the most important of which is c-Jun, activated by phosphorylation on Ser 63 and 73 (27). ATF-2 and Elk-1 are also activated by JNKs (21), (12). Transcriptional activity enhanced by these proteins is responsible for cells biologic response. JNK2 and JNK3 seem to be responsible for the activation of transcription factors whereas the basal constitutive presence of activated JNK1 is not effective in phosphorylation of transcription factors including c-Jun (13), (14). c-Jun phosphorylation was also linked to the protection or regeneration (19) and it has not been clarified if JNK can also contribute to pro-survival action.

Regarding cytoplasmic substrates, it has been shown that there is interdependency between JNK signaling and mitochondrial apoptosis (31). Oxygen-glucose deprivation in hippocampal neurons from Jnk3-null mice compared to WT mice suggests a critical JNK3 role in both the apoptotic process and the release of cytochrome c from mitochondria (24). The release of cytochrome c from mitochondria is controlled by specific members of the Bcl-2 family protein, whose phosphorylation could be regulated by JNKs (28), (35).

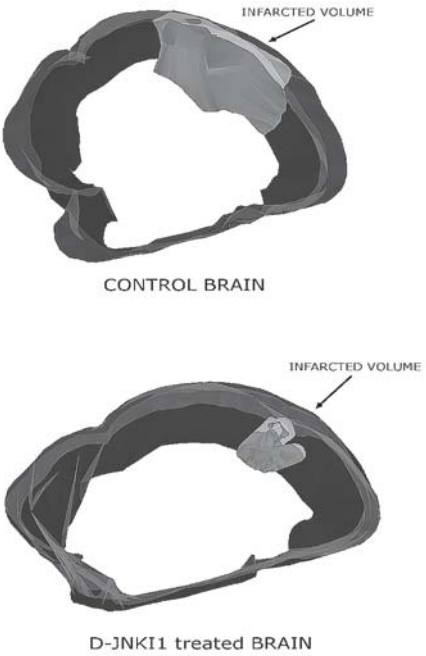

Figure 4. Three-dimensional reconstruction showing examples of lesion in control rat and D-JNKI-1 treated rat 6 hours after permanent ischemia (middle cerebral artery occlusion). This ischemic lesion results in a unilateral degeneration of the parietal cortex 24 hours following ischemia. The areas of the ischemic lesion and of the whole brain were traced using the Neurolucida software program for computer-aided microscopy.

DENN/MADD is also a JNK cytoplasmic substrate implicated in cell death. It has a death domain and was identified as a substrate for JNK3, which is the isoform mostly present in the CNS (41). The meaning of this phosphorylation is still unclear, but increasing evidences support a correlation between low MADD expression and neuronal loss: it has recently been shown that MADD down-regulation correlates with neuronal cell death in

Alzheimer's disease brain and hippocampal neurons (15).

Finally, JNK is involved in the phosphorylation of Tau and APP, two proteins strongly related to neurodegenerative diseases. In fact, hyperphosphorylation and accumulation of Tau in neurons (and glial cells) is one of the main pathologic hallmarks in Alzheimer's disease and other tauopathies. Many of the hyperphosphorylated sites are serine/threonine-proline sequences. Recently it has been shown that all three JNK isoforms phosphorylate Tau at many serine/threonine-prolines, as assessed by the generation of the epitopes of phosphorylation-dependent anti-Tau antibodies (40). Phosphorylation by JNK isoforms resulted in a greatly reduced ability of Tau to promote microtubule assembly, which would compromise neuronal transport and would result in neuronal dysfunction.

APP is efficiently phosphorylated in its cytoplasmic region by JNK3 in vitro (33) and APP phosphorylation by JNK is enhanced by the association of APP with scaffold protein JIP-1b (20). Moreover, an enhanced activation of JNK pathway and an attenuation of apoptosis by SP600125, the ATP competitive JNK inhibitor, through protection of mitochondrial dysfunction and reduction of caspase 9 activity have been demonstrated (25). These results put in evidence that JNK could play an important role in the pathogenesis of Alzheimer's disease.

CONCLUSIONS

JNK could provide a suitable target for the protection against neuronal loss and considerable effort is being directed to the development of JNK inhibitors. This resulted in a couple of chemical inhibitors: CEP-1347, an inhibitor of the MLK family of JNK pathway activators, and SP600125, a direct inhibitor of JNK activity. These commonly used inhibitors have demonstrated efficacy for use in vivo, with the successful intervention in animal models. An alternative approach is represented by the cell permeable peptide D-JNKI-1, which is a new powerful neuroprotective agent. D-JNKI-1 is an efficient and specific inhibitor of JNK action. It does not inhibit JNK's enzymatic activity, like the classical small chemical inhibitors, but it selectively blocks access to many of its substrates by a competitive mechanism and results as the most selective inhibitor proving its exceptional superiority.

D-JNKI-1 protects against several forms of excitotoxicity in vitro (8), (9), and it confers the strongest protection even reported before against ischemic cell loss in vivo with extended therapeutic window, since it still protects when administered 6-12 hours after the ischemic insult (8). Moreover it has been shown that it is really able to completely prevent the death program and not just to delay it, as a significant protection was still seen after one or two weeks following ischemic lesion in transient ischemia: this confirms that it could be a promising agent to obtain neuroprotection in stroke. D-JNKI-1 protective action has also been tested with positive results in two other models of CNS injury, hair cell death and hearing loss following ototoxic drug or acoustic trauma exposure (36) and retinal ganglion cells death following optic nerve crush (34): these results provide strong evidence that JNK inhibition with D-JNKI-1 could be a very interesting therapeuthic approach to prevent cell loss in the CNS.

Despite these promising results further studies will be necessary in order to detect D-JNKI-1 side effects, and caution is required in view of JNK's involvement in metabolic

regulation and neuronal plasticity and regeneration. In fact, it has to be admitted that the underlying cell biology of this powerful neuroprotection is still largely unknown.

A deep understanding of the organization and function of the JNK signaling cascade will be crucial to improve the specificity of the JNK inhibitors. In fact, due to the complex cross-talk within different signaling cascades, as well as peculiar neuronal response, it is difficult to predict potential adverse events that might arise from JNK inhibition. However, it appears clear that further studies are needed to offer greater specificity in order to prevent only the pathological responses of this signaling, that extent its physiological functions in such a large range.

Detailed studies about D-JNKI-1 toxicity and its potential side effects are now essential. Until these aspects are clarified the question will remain if D-JNKI-1 could represent an important molecule in human therapy and whether it can be extended to clinical trails.

ACKNOWLEDGEMENTS

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Chapter 14

SALVAGE OF ISCHEMIC MYOCARDIUM: A FOCUS ON JNK

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Abstract: Myocardial infarction is a problem of utmost clinical significance, associated with an important morbidity and mortality. Actual treatment of this affection is focusing on the reperfusion of the occluded coronary-artery. A complementary approach would be to prevent the death of the ischemic myocardium by interacting with detrimental intracellular pathways. Several strategies have been successfully used to reduce the size of myocardial infarction in animal models. In this article, we will focus on the c-Jun N-terminal kinase (JNK), a member of the mitogen-activated (MAPK) protein kinase family and an important determinant of cell survival/death. We will review the role of JNK in cardiac ischemia/reperfusion and summarize recent advances in the use of JNK inhibitors to protect the myocardium.

Key Words: myocardial infarction, ischemia/reperfusion, treatment, JNK, MAPK

INTRODUCTION

Myocardial infarction is a problem of utmost clinical significance, associated with an important mortality and morbidity. In 2002, over 850000 persons suffered from a myocardial infarction in the United States, with an associated mortality of roughly 25%. The resulting annual economical burden was estimated at 142 billion dollars (2). Since the prevalence of cardiovascular diseases is rapidly increasing in developing countries, ischemic heart disease is becoming one of the leading causes of mortality worldwide (1).

Initially, treatment of myocardial infarction was limited to bed rest and fighting potential complications. In a pioneering editorial in 1974, E. Braunwald wrote:"… it is now fair to accept the position that just because myocardial tissue lies within the distribution of a recently occluded coronary artery does not mean that it is necessarily condemned to death."

Hypoxia and Exercise, edited by R.C. Roach *et al.*
Springer, New York, 2006.

(8) Most of the ensuing work, aimed at rescuing the ischemic myocardium, focused on coronary artery reperfusion, leading to a dramatic improvement of the clinical outcome after myocardial infarction. More recently, a novel focus of intense research is the prevention myocardial cell death during ischemia. This approach could be complementary to reperfusion.

CARDIAC PROTECTION STRATEGIES

One of the earliest attempts to protect the ischemic myocardium was the treatment with glucose-insulin-potassium infusions. The rationale was to provide metabolic support to the cardiomyocytes by shifting the source of myocardial energy from free fatty acid oxidation (FFA) to glucose oxidation, rendering the heart more "oxygen effective". Furthermore, during acute myocardial infarction, FFAs levels rapidly increase because of lipolytic effects of catecholamines, in turn leading to membrane damage. Treatment with glucose-insulin-potassium was able to decrease circulating levels of FFA and in turn protecting the myocardial cells. However, so far, clinical studies with glucose-insulin-potassium infusions after myocardial infarction provided highly controversial results regarding the benefits of this adjunctive therapy (4). Recently, a double-blind prospective study including more than 20000 patients with acute myocardial infarction showed no benefit of glucose-insulin-potassium infusion on mortality, cardiac arrest or cardiogenic schock (26).

Interestingly, the beneficial effects of the beta-blocker carvedilol could also be related to a direct effect on the cardiomyocytes. Indeed, in animal models of ischemia/reperfusion, carvedilol was able to directly prevent cell death of cardiomyocytes (40).

Due to the clinical significance of ischemic heart disease, several cardioprotective strategies are currently under investigation in animal models of myocardial ischemia/reperfusion. The common goal of these therapies is to interfere with intracellular pathways in order to promote cell survival and/or reduce cell death. Successfully reducing infarct size in animal models was achieved with a number of treatment such as erythropoietin (10), rosiglitazone (36) and even carbon monoxide (19), for example.

NECROSIS AND APOPTOSIS

Two distinct types of cell death occur in the myocardium, namely necrosis and apoptosis. The mechanisms underlying the development of cell necrosis are still poorly understood. Originally considered as a passive cellular event, data obtained in C. elegans suggests that necrosis involves activation of specific, energy-dependant cellular pathways (38).

On the other hand, the cellular and molecular mechanisms leading to apoptosis have been studied extensively in the last decades. Apoptosis is a highly regulated, energy-dependent form of cell death. Central to apoptosis are the disabling of mitochondrial function and the activation of caspases, a subclass of cysteine proteases, ultimately leading to proteolytic destruction of the cell (35). In vivo, apoptosis of cardiac myocytes is present after ischemia (32) especially when followed by reperfusion (18), heart failure (28) and viral myocarditis (23).

Originally only necrosis was thought to mediate ischemic cell death. To date, the rela-

tive contributions of apoptosis and necrosis during ischemia/reperfusion are not clearly established. However, the importance of apoptosis have been highlighted by demonstrating that genetic manipulations aimed at inhibiting apoptosis in mice resulted in a 60 to 70% reduction of the size of the myocardial infarction following ischemia/reperfusion (9) (25).

THE JNK PATHWAY

During the course of life, the heart has to adapt to a series of physiological and pathological stresses, such as exercise, hypoxia, pressure overload, ischemia or infections. Several regulatory pathways are critical signal transducers involved in the dynamical adaptation of the myocardium to stresses. Amongst them are the mitogen-activated protein kinases (MAPKs). MAPKs are serine-threonine protein kinases that are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence; acting as central regulators of cell survival, growth and transformation (37). The MAPK activation module includes three levels of kinases, comprising a MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK, leading to sequential activation of the downstream target. In mammals three families of MAPK have been identified: the c-Jun N-terminal kinase (JNK), the extracellular signal-regulated protein kinase (ERK 1/2, ERK 3/4, ERK 5 or big MAPK-1) and p38.

The JNK proteins are encoded by three different genes. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is mostly restricted to brain but seems to be also found in testis, and very weakly in the heart. Two different transcripts results in the formation of a 46 kDa and 55 kDa isoform for each JNK gene, but the functional significance of these splice variants is still unclear. Mice with genetically disruption of the genes encoding for JNK1 or 2 appear morphologically normal but are immunodeficient due to T cell function defects (12).

Activation of JNK starts at the cell membrane where small GTP-binding proteins activate MAPK kinase kinase 1, 2, 3 (MEKK 1, 2, 3) and apoptosis signal-regulating kinase 1 (ASK1) in turn phosphorylating MAPK kinase 4 and 7 (MKK4 and 7), resulting in direct JNK activation. Since protein-protein interactions are important for JNK activation, the assembly of the JNK module by scaffold proteins enables efficient JNK phosphorylation. The protein islet-brain 1 and 2 (IB-1 and -2), also called JNK interacting protein (JIP 1 and 2) are molecular scaffolds bringing the different component of the module in close proximity and therefore facilitating JNK activation (12). These proteins are highly expressed in the brain and insulin-secreting cells of the pancreas. Lowers levels are also found in the heart, ovary and small intestine.

Although the activation of JNK during myocardial ischemia has not been clearly proven, it is now well established that JNK is dramatically activated during reperfusion (5; 22). In rodent models of ischemia-reperfusion, cardiac JNK activation starts as early as 1 minute post reperfusion and lasts up to 3 hours (27). In a rat model of myocardial infarction, increased JNK activity was present after 15 min and returned to normal after 24 hours (34). Finally in humans, JNK activation has been demonstrated in patients with heart failure secondary to ischemic heart disease (11). Regarding the mechanisms of JNK activation during ischemia/reperfusion, it appears that increased oxidative stress plays a major role. In cultured rat neonatal myocytes, exposure to hypoxia/reoxygenation results in a dramatic

increase in the generation of reactive oxygen species that is associated with JNK activation. Pretreatment with antioxidant could prevent this activation (14). In a perfused rat heart model, treatment with superoxide dismutase or catalase was able to prevent, at least in part, ischemia/reperfusion-induced JNK activation (22).

Regarding the significance of JNK activation on cell survival, studies in cultured cardiomyocytes have given equivocal results. While some authors found that activation of JNK promoted to apoptosis (3; 20), another report showed that activated JNK protected the cardiomyocytes after oxidative stress (15). In contrast, cardiac JNK activation in vivo is clearly detrimental. In mice, targeted cardiac activation of JNK due to transgenic, cardiac-restricted expression of a constitutively active mutant of MMK7 induced the induction of the fetal gene program and the development of congestive heart failure, associated with conduction defects (30). In line with this concept, preventing the activation of JNK due to oxidative-stress resulted in reduction of apoptosis (24). Similarly, treatment with carvedilol prevented the ischemia/reperfusion-induced activation of JNK and decreased apoptosis (40).

Over the past several years, various epidemiological studies have strongly suggested that mild-to-moderate alcohol consumption, in particular red wine, is associated with a reduced incidence of mortality and morbidity from coronary heart disease (The French Paradox) (31). Interestingly, the grape seed proanthocyanidin has been shown to prevent, at least in part, ischemia/reperfusion-induced JNK activation and to decrease cardiomyocytes apoptosis and prevent left-ventricular dysfunction in a perfused heart model of ischemia/reperfusion (33).

Several downstream mechanisms may explain the pro-apoptotic effects of activated JNK. It has been demonstrated that during ischemia/reperfusion activated JNK was associated with mitochondria (20), where it induced cytochrome C release, consequently promoting apoptosis (3). Furthermore anti-apoptotic proteins from the Bcl-2 family were found to be phosphorylated by JNK, leading to their inactivation (39). JNK also directly phosphorylates and activates the pro-apoptotic factor Bad (13).

Taken together these data show that ischemia/reperfusion-induced activation of cardiac JNK leads to cardiomyocytes apoptosis and provide the rational for JNK inhibition as a mean to protect the myocardium from ischemia/reperfusion injury.

INHIBITION OF JNK

In cultured rat neonatal cardiomyocytes, inhibition of JNK1, but not JNK2 translation with gene specific antisense oligonucleotides, resulted in a 50% decrease of the number of apoptotic cells (21). In vivo, in a rat model or ischemia/reperfusion, the non-peptidic JNK inhibitor AS601245 has been shown to decrease infarct size by 40% when given during the whole reperfusion period (Figure 1). The effect was associated with 85% reduction in JNK activation, whereas the other member of the MAPKs appeared to be unaffected by the treatment (17).

However, one of the issues that may occur with chemical inhibitors is their lack of specificity and potential side effects. To circumvent this problem a novel approach to JNK inhibition was developed. JNK interacts with its substrate via a 15-20 amino acids sequence called JNK-binding domain (JBD). For example, JIP-1/IB1 and c-Jun share a simi-

lar binding motif, but JNK's affinity of binding to JIP-1/IB1 is about 100-fold higher. It has been demonstrated that expression of the IB1-derived binding domain could selectively block the access of JNK to c-Jun and other substrates by a competitive mechanism, and in turn prevent its detrimental effects (29). To make this novel peptide cell permeable, the 10-amino-acid HIV Tat transporter peptide was fused to the 20-amino-acid JNK-binding motif of JIP-1/IB1. In addition, it was synthesized as a protease-resistant *all-D-retroinverso* form (D-JNKi) to expand its half-life *in vivo*. In vitro, this peptide protects insulin-secreting TC-3 cell lines against IL-1-induced apoptosis (6). In a murine stroke model, intraventricular administration of the peptide reduced the size of the lesion by 90% and prevented long-term behavioral consequences (7).

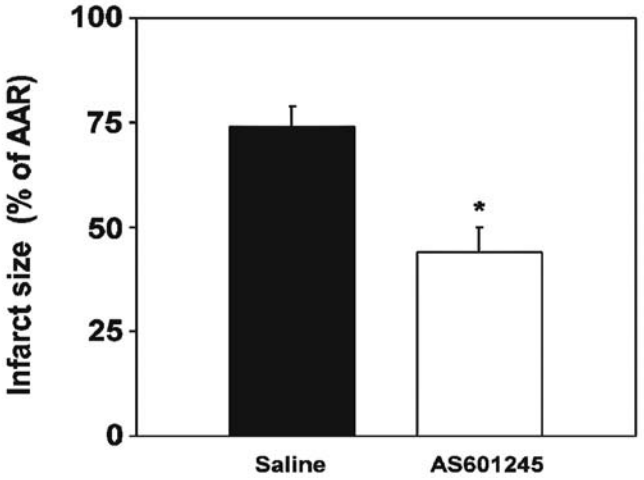

Figure 1. Reduction of myocardial infact size by AS601245.

With these data at hand, we tested the effects of D-JNKi in a mouse model of cardiac ischemia/reperfusion. Our preliminary results show that intraperitoneal treatment with D-JNKi, when administered prior to ischemia significantly reduced the size of the infarct by roughly 70%. Most interestingly, even when administered after ischemia and reperfusion, this peptide significantly reduced, albeit to a lesser extend, the size of the infarct (16). Therefore, these very promising data give us the rationale for the use of specific peptide inhibitors of JNK as potential treatment of myocardial infarction (Figure 2).

FUTURE PERSPECTIVES

Advances in molecular medicine have enabled us to identify critical pathways involved in cell survival or death in the myocardium. Furthermore, critical regulators of these pathways have been identified, leading to the development of novel therapeutic approaches of ischemic heart disease. Preliminary results in murine models in vivo are promising, however further studies are granted before a clinical application, where coronary-artery reperfusion and prevention of cardiomyocytes death will be the gold standard of the treatment of myocardial infarction.

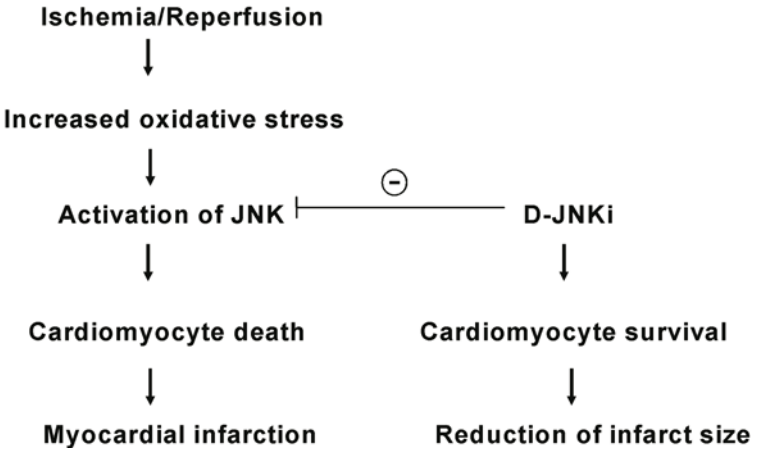

Figure 2. JNK-mediated cell death during myocardial ischemia/reperfusion and effects of D-JNKi, a novel peptide inhibitor of JNK.

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Chapter 15

MITOCHONDRIAL REACTIVE OXYGEN SPECIES ARE REQUIRED FOR HYPOXIC HIFα STABILIZATION

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Abstract: Multicellular organisms initiate adaptive responses when oxygen (O_2) availability decreases. The underlying mechanisms of O_2 sensing remain unclear. Mitochondria have been implicated in many hypoxia-inducible factor (HIF) –dependent and –independent hypoxic responses. However, the role of mitochondria in mammalian cellular O_2 sensing has remained controversial, particularly regarding the use pharmacologic agents to effect hypoxic HIFα stabilization, which has produced conflicting data in the literature. Using murine embryonic cells lacking cytochrome c, we show that mitochondrial reactive O_2 species (ROS) are essential for O_2 sensing and subsequent HIFα stabilization at 1.5% O_2. In the absence of this signal, HIFα subunits continue to be hydroxylated and degraded via the proteasome. Importantly, exogenous treatment with H_2O_2 and severe O_2 deprivation is sufficient to stabilize HIFα even in the absence of functional mitochondrial. These results demonstrate that mitochondria function as O_2 sensors and signal hypoxic HIFα stabilization by releasing ROS to the cytoplasm. The cytochrome c mutant embryonic cells provide a unique reagent to further dissect the role of mitochondria in O_2 mediated-intracellular events.

Key Words: transcription, signal transduction, HIF prolyl hydroxylases

INTRODUCTION

Molecular oxygen is essential for aerobic energy metabolism, whereby the oxidoreduction energy of mitochondrial electron transport is converted into the high-energy phosphate bond of ATP. Oxygen (O_2) serves as the final electron acceptor for electron transport at complex IV (cytochrome c oxidase). However, where excess O_2 can be toxic, a significant decrease in O_2 impairs ATP generation and cell viability. Therefore, most prokaryotic and

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eukaryotic life is restricted to a narrow range of pO_2. Most mammalian cells exist at an *in vivo* pO_2 of 30 mm Hg to 50 mm Hg (4-7% O_2). As O_2 levels drop below this range, a number of cellular responses are engaged, including inhibition of ion channel activity, cell division, ribosome biogenesis, and mRNA translation. Cells have also developed transcriptional responses to hypoxia mostly regulated by hypoxia-inducible factors (HIFs) (12). HIFs activate transcription of genes encoding proteins that enhance glycolytic ATP production or increase O_2 delivery to affected tissues. This review will focus on models of O_2 sensing and their impact on the HIF signaling pathway (Figure 1).

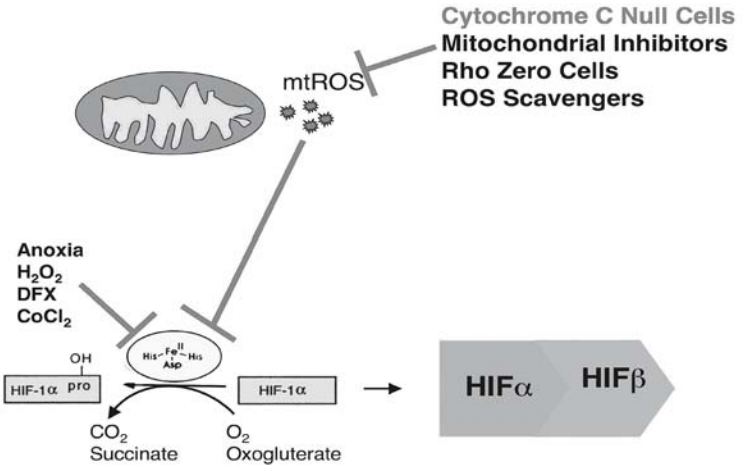

Figure 1. Proposed role of mitochondria in cellular oxygen sensing. This figure documents the experimental evidence supporting a role for mitochondrial reactive oxygen species (ROS) in the modulation of HIF stability, including cytochrome c null cells, mitochondrial inhibitors, Rho 0 cells, and ROS scavengers. We believe that mitochondrial ROS inhibit prolyl hydroxylase enzymatic activity in a similar fashion to anoxia, H_2O_2, DFX, and cobalt chloride. Inhibition of hydroxylase activity leads to HIFα accumulation, dimerization with HIFα (ARNT), and HIF-mediated gene expression.

OXYGEN SENSORS AND HYPOXIC SIGNAL TRANSDUCTION

The mechanisms by which eukaryotic cells sense decreased O_2 and transduce this signal to the HIF pathway have been debated for at least a decade. HIFs are heterodimeric transcription factors consisting of alpha (HIF-1α, HIF-2α, and HIF-3α), and beta (ARNT, ARNT2) subunits. The beta subunits are constitutively transcribed and translated and reside in the nucleus. In contrast, while the alpha subunits are transcribed and translated at a high rate in normoxic conditions (21% O_2), they are rapidly degraded via the proteasome. HIF-1α, HIF-2α, and HIF-3α contain 200 amino acid "oxygen dependent degradation domains" (ODDs) (5). These ODDs include a highly conserved binding domain for the tumor suppressor Von Hippel-Lindau protein (pVHL). pVHL coordinates the assembly of an E3 ubiquitin ligase complex containing Elongin B, Elongin C, Cullin 2, and RBX 1, and this complex ubiquitylates HIFα's, targeting their degradation via the proteasome (6) (7).

Recent data have shown that the interaction between HIFα's and pVHL is regulated via hydroxylation of proline residues in the ODD (6) (7). There are at least three HIF prolyl hydroxylases in mammalian cells (4). In the absence of O_2, prolyl hydroxylases are inactive; the unmodified HIFα no longer interacts with pVHL and accumulates. The absolute requirement for O_2 by HIF prolyl hydroxylases indicates that these enzymes may function as direct oxygen sensors. However, the precise sensor for the HIF pathway remains contentious. Previous models have included heme-binding proteins, membrane associated NADPH oxidases, and mitochondrial reactive oxygen species (10).

The model based on NADPH oxidase was first proposed in 1996 (8) (15). NADPH oxidases convert O_2 into reactive oxygen species (ROS); decreased pO_2 would therefore result in *reduced* formation of ROS to stabilize HIFα subunits. However, oxygen sensing and HIFα stabilization during hypoxia are preserved in gp91-*phox*-deficient cells isolated from knockout mice or patients with chronic granulomatous disease (1). These and other observations have shed doubt on a role for NADPH oxidase in oxygen sensing and HIF activation. A third model proposed in 1998 by Chandel et al. (2) suggests that hypoxia results in *increased* production of ROS by the mitochondria. Here, linear decreases in pO_2 result in a progressive increase in ROS. Pharmacological inhibitors of different complexes of the mitochondrial respiratory chain support the hypothesis of mitochondria as a key intracellular source of ROS. Inhibition of complex I (via rotenone) suppresses ROS production while inhibition of complex III or IV (antimycin A) increases ROS. Moreover, HIF activation directly correlates with ROS accumulation. Antioxidants such as ebselen and PDTC attenuate the ROS signal and abolish HIF stabilization. Importantly, Rho 0 cells (where mitochondria are deficient in electron transport and oxidative phosphorylation) fail to induce HIF activity. These results were not reproduced using other cell types: Srinivas et al. (13) and Vaux et al. (14) observed that Rho 0 cells display a normal response to hypoxia based on HIF stabilization. Vaux et al. used mutant cell lines with specific genetic defects in the electron transport chain and demonstrated that HIF activation by hypoxia was preserved. Taken together, these results failed to support the model implicating mitochondrial ROS during hypoxia as effectors in HIFα stabilization. However, Schroedl et al. accounted for this discrepancy by comparing HIFα stabilization under hypoxic conditions (1.5% O_2) versus anoxic conditions (less than 0.1% O_2) (11). Both the Srinivas and Vaux studies observed hypoxic HIFα stabilization using extremely low levels of O_2. The Schroedl et al. paper showed that mitochondrial ROS are essential for HIF stabilization at 1% O_2, while extremely low levels of O_2 (less than 0.01% O_2) are independent of mitochondrial electron transport. These results provide a plausible explanation, as the HIF prolyl hydroxylase enzymes are dependent on O_2 as a cofactor and at anoxic conditions could become substrate limited.

Both of these models suggest ROS are key players in O_2 sensing by the HIF pathway. However, the effector(s) regulated by ROS that transduces the signal to HIF remain unknown. Further investigation is also required to identify these intermediates and determine if they modulate prolyl hydroxylase enzymatic activity.

CYTOCHROME C IS REQUIRE FOR CELLULAR OXYGEN SENSING AND HYPOXIC HIFA ACTIVATION

While cellular responses to hypoxia have been studied extensively, the precise identity of mammalian cellular O_2 sensors remains controversial. Because of their O_2 dependence and relatively high kM for O_2 *in vitro*, it has been proposed that the HIF prolyl hydroxylases directly sense O_2 deprivation to stabilize HIF. However, mitochondrial ROS have been implicated in many cellular processes including HIFα stability and transcriptional activity, myocyte contraction, IL-6 production, glutathione depletion, NA,K-ATPase activity, and adipocyte differentiation (15). Despite this evidence, the role of mitochondria in mammalian cellular oxygen sensing has remained controversial. The use of pharmacological agents to affect HIFα stabilization has produced conflicting data in the literature. Therefore, we investigated this controversy by repeating and extending previous observations.

Mitochondria-deficient Hep3B Rho 0 cells have been shown to be defective in HIFα stabilization (3). Nevertheless, later work suggests this may be an artifact due to their selection with the mitochondrial inhibitor rotenone (14). We initially chose to address this discrepancy by generating Hep3B Rho 0 cell using ethidium bromide and avoiding selection with mitochondrial inhibitors (9). Of note, Hep3B cells exposed to steady doses of ethidium bromide for 3 weeks exhibit no mitochondrial DNA or oxygen consumption. Upon exposure to 1.5% O_2, Rho 0 cells failed to stabilized either HIF-1α or HIF-2α. We also generated HEK293 Rho 0 cells and used human osteosarcoma-derived 143B Rho 0 cells and obtained similar results. Hep3B, HEK293, and 143B cells retained responsiveness to desferrioxamine (DFX) with regard to HIF stabilization. These findings indicate that mitochondrial electron transport is required for hypoxic HIFα stabilization at 1.5% O_2, indicating that all cells retained active prolyl hydroxylation.

Due to concerns about long-term metabolic adaptation or genetic alteration of Rho 0 cells, we also employed acute mitochondrial inhibition of complex I (rotenone) or complex III (myxothiazol), minimizing non-specific effects by utilizing extremely low nanomolar doses. Treatment of Hep3B cells with rotenone or myxothiazol inhibited respiration and prevented hypoxic HIF-1α and HIF-2α stabilization after 2 hours of treatment. Importantly, myxothiazol treatment also affected HIFα stabilization in response to cobalt chloride, especially at higher doses. These results highlight difficulties inherent to direct pharmacological treatment and have prevented widespread acceptance of mitochondrial ROS during mammalian O_2 sensation.

We, therefore, employed a novel genetic model to further investigate the role of mitochondrial ROS in cellular oxygen sensing avoiding the non-specific effects of ethidium bromide or pharmacological inhibition all together. The murine somatic cytochrome c gene has been targeted previously. Loss of cytochrome c prevents oxidation of cytochrome c1, keeping the Reiske's iron sulfur protein reduced. This prevents oxidation of ubiquinol and the formation of the ubisemiquinone radical, eliminating one important source of superoxide anion (O_2^-) (and therefore H_2O_2) that has been implicated in hypoxic HIFα stabilization. We generated wild type, heterozygous, and null cell lines for cytochrome c using day 8.5 mouse embryos. While wild type and heterozygous cells exhibit similar respiratory rates while null cells are devoid of any measurable mitochondrial O_2 assumption or cytochrome c protein.

We next investigated *in vivo* ROS production in the cells using oxidant sensitive dichlorofluorescein (DCFH) and a ROS-sensitive FRET probe. Where wild type and heterozygous cells rapidly and dramatically increase oxidation and FRET activation, cytochrome c null cells exhibit an attenuated response. Importantly, unlike their wild type or heterozygous

counterparts, cytochrome c null cells are unable to stabilize HIF-1α or HIF-2α in response to treatment with 1.5% O_2. The null cells, however, retain an appropriate response to DFX. These results argue strongly that the increased ROS production in hypoxic heterozygous cells are mitochondrial, and supports the hypothesis that mitochondrial ROS generated by complex III are responsible for hypoxic HIF-1α and HIF-2α stabilization.

Stable reintroduction of cytochrome c into an independent null cell line restores mitochondrial function and hypoxic mitochondrial ROS accumulation. This correlated with restoration of properly stabilized HIF-1α and HIF-2α levels similar to wild type cells. We concluded that the inhibility of null cells to properly sense O_2 is due to the lack of a functional mitochondrial electron transport chain resulting from cytochrome c loss. All cell lines respond similarly to DFX, suggesting that cytochrome c null cells maintain normal HIFα transcription, translation, and degradation even in the absence of functional mitochondrial. However, to test that they continue to hydroxylate and degrade HIF-1α and HIF-2α under hypoxic conditions, cytochrome c wild type and null cells were treated with 1.5% O_2 in the presence of the proteasome inhibitor MG132. While the null cells failed to stabilize HIFα in response to hypoxia, inhibition of proteasome degradation by MG132 leads to HIFα accumulation similar to data obtained with wild type cells. This demonstrates that hydroxylation and degradation of HIFα continue to occur in the cytochrome c null cells even at this reduced O_2 concentration.

We next determined if HIFα stabilization can be bypassed by severe O_2 deprivation (anoxia or approximately 0% O_2) where prolyl hydroxylases appear to be substrate limited. Importantly, the null cells fail to stabilize HIF-1α or HIF-2α in response to 1.5% O_2. However, exposure to 0% O_2 induces both HIF-1α or HIF-2α in a mitochondria-independent manner. These results suggest functional mitochondria are not necessary for cellular responses to severe O_2 deprivation. To test whether ROS production is also sufficient to stabilize HIF-1α or HIF-2α, Hep3B cells were treated with exogenous hydrogen peroxide (H_2O_2) as previously described (3). This resulted in normoxic stabilization of HIF-1α or HIF-2α similar to that induced by hypoxia in the same period of time. Furthermore, incubation of Hep3B cells with an H_2O_2 generating enzyme, glucose oxidase, is sufficient to stabilize both HIFα subunits. This is shown to be H_2O_2-dependent as inclusion of catalase abolishes HIFα stabilization. Finally, treatment of cytochrome c null cells with T-butyl hydroperoxide (TBP) a stable H_2O_2 analog, also induces HIFα stabilization similar to the hypoxia mimetic cobalt chloride. These results indicate that ROS production is sufficient for HIFα stabilization and likely functions downstream of mitochondria.

In summary, cytochrome c null cells provide novel genetic evidence supporting a role for mitochondrial ROS in the mammalian O_2 sensing pathway that leads to HIFα stabilization. How mitochondrial ROS effect HIF prolyl hydroxylase activity is important to understanding cellular O_2 sensing. These experiments are ongoing in the laboratory and suggest that ROS may directly inhibit the prolyl hydroxylases perhaps by oxidizing Fe^{2+} at the active site. It is likely that a number of mammalian cellular O_2 sensor exist, controlling various hypoxic responses depending on the cell type, O_2 tension, and other cellular conditions. The cytochrome c null cells provide valuable tools for investigating the role of the mitochondria and other hypoxic responses such as the inhibition of protein synthesis and cell cycle progression.

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Chapter 16

HYPOXIA-INDUCED GENE ACTIVITY IN DISUSED OXIDATIVE MUSCLE

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Abstract: Hypoxia is an important modulator of the skeletal muscle's oxidative phenotype. However, little is known regarding the molecular circuitry underlying the muscular hypoxia response and the interaction of hypoxia with other stimuli of muscle oxidative capacity. We hypothesized that exposure of mice to severe hypoxia would promote the expression of genes involved in capillary morphogenesis and glucose over fatty acid metabolism in active or disused soleus muscle of mice. Specifically, we tested whether the hypoxic response depends on oxygen sensing via the alpha-subunit of hypoxia-inducible factor-1 (HIF-1α). Spontaneously active wildtype and HIF-1α heterozygous deficient adult female C57Bl/6 mice were subjected to hypoxia (PiO_2 70 mmHg). In addition, animals were subjected to hypoxia after 7 days of muscle disuse provoked by hindlimb suspension. Soleus muscles were rapidly isolated and analyzed for transcript level alterations with custom-designed AtlasTM cDNA expression arrays (BD Biosciences) and cluster analysis of differentially expressed mRNAs. Multiple mRNA elevations of factors involved in dissolution and stabilization of blood vessels, glycolysis, and mitochondrial respiration were evident after 24 hours of hypoxia in soleus muscle. In parallel transcripts of fat metabolism were reduced. A comparable hypoxia-induced expression pattern involving complex alterations of the IGF-I axis was observed in reloaded muscle after disuse. This hypoxia response in spontaneously active animals was blunted in the HIF-1α heterozygous deficient mice demonstrating 35% lower HIF-1α mRNA levels. Our molecular observations support the concept that severe hypoxia provides HIF-1-dependent signals for remodeling of existing blood vessels, a shift towards glycolytic metabolism and altered myogenic regulation in oxidative mouse muscle and which is amplified by enhanced muscle use. These findings further imply differential mitochondrial turnover and a negative role of HIF-1α for control of fatty acid oxidation in skeletal muscle exposed to one day of severe hypoxia.

Key Words: HIF-1α, microarray, angiogenesis, altitude, ischemia

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INTRODUCTION

Ever since the early observations on ischemia and altitude exposure, hypoxia has been seen as an important modulator of the muscular phenotype (reviewed by 1). Subsequent studies laid out a picture whereby hypoxia alone may affect glycolytic flux, protein synthesis and mitochondrial oxidative capacity (18,29).

In fact, skeletal muscle is a particular hypoxia-sensitive tissue as muscular oxygen tension is subject to large variations during daily use. Rapidly after the onset of exercise, a significant 2-fold drop in muscular oxygen tension is apparent (reviewed by 20, 36). Similarly, muscle pO_2 drops to low levels in consequence of reduced oxygen pressure of the inspired air (32, 41). Concomitant exercise additionally lowers the oxymyoglobin concentration in muscle (36).

While this hypoxia-based drop in oxygen tension certainly represents an important stimulus, it has been argued that many of the muscular adjustments to hypoxia such as alterations in fiber size and aerobic enzymes depend on a number of additional factors. These co-variables include the level of exercise and hypoxia (4). These studies also pointed out that hypoxia alone does not alter capillary number (26). Endurance exercise in fact, exerts pronounced control over similar aspects of muscular processes as the hypoxic stimulus. For instance, endurance training is known to ameliorate energy metabolic processes mainly via enhancing mitochondrial oxidative capacity and increasing capillarity (17).

The understanding of the molecular mechanisms underlying hypoxia-dependent muscular adaptations and the interaction with exercise stimuli is still incomplete. Altered expression of gene messenger, i.e. mRNAs, has been recognized as a possible strategy involved in the integration of the hypoxia stimuli into a phenotypic response. Several hypoxia sensitive mRNAs have been identified in muscle tissue. The recent systematic analysis of the expressional response of cultured endothelial cells and skeletal muscle to hypoxia or ischemia indicates that the mRNA response is more complex than previously assumed. These changes involve specific alterations of the total pool of RNAs, i.e. the transcriptome (30, 31). With respect to trans-activation of hypoxia-dependent genes, the HIF-1 system has been recognized to be crucial for hypoxia sensing in various systems (14, 38). This master switch is a dimeric complex, termed hypoxia-inducible factor-1 (HIF-1), the α subunit of which is tagged for degradation in normoxia. Conversely, HIF-1α is rapidly stabilized in severe hypoxia thereby causing its association with the HIF-1β subunit to form the DNA-binding HIF-1 complex which initiates transcription of various oxygen responsive genes (40). HIF-1α therefore promotes angiogenesis and a shift towards glycolysis in conditions of ischemia (14, 38). The importance of HIF-1α in the hypoxia response is underlined by the observation that HIF-1α null mice die at midgestation and that ablation of one HIF-1α allele produces defective tissue response in situations where muscular oxygen tension is suspected to drop (25, 38). For instance, multiple systemic defects to chronic hypoxia (10% O_2) involving polycythemia, right ventricular hypertrophy, pulmonary hypertension, and defect pulmonary vascular remodeling are apparent in HIF-1α heterozygous mice (45). Analogously, the partial ablation of the HIF-1α gene in conditional knock-out mice produces measurable muscular aberrations. These involve a metabolic shift away from glycolysis towards enhanced capacity for the mitochondrial Krebs cycle and beta oxidative enzymes thereby improving exercise endurance (25).

We have developed a muscle-specific cDNA microarray to map the transcriptome al-

terations which underlie the structural adjustments with disuse of mouse soleus muscle in the hindlimb suspension-reloading model (6). The current exploration applied this technology in combination with transgenic technology to identify gene expressional alterations in the soleus muscle of mice induced by short term severe hypoxia. Additionally, we intended to reveal the interaction of the hypoxia response in this anti-gravitational muscle with mechanical stimuli and to identify the underlying hypoxia-sensing mechanism. It was hypothesized that exposure of mice to severe hypoxia (PiO_2 70 mmHg) for 24 hours would promote a distinct enhancement of expression for genes involved in capillary morphogenesis and glucose metabolism over the combustion of fatty acids in the oxidative soleus muscle. We tested whether this expressional response depends on sensing via HIF-1α by subjecting wildtype and HIF-1α heterozygous deficient adult female C57Bl/6 mice to hypoxia. Alternatively, animals were subjected to hypoxia after 7 days of muscle disuse provoked by hindlimb suspension to reveal whether the hypoxia signature of gene expression would be preserved when the hypoxia stimuli was combined with mechanical stress.

METHODS

Spontaneously active mice: 3-4 months old wildtype (wt) or HIF-1α heterozygous deficient (HIF-1α+/-) female mice (C57BL/6) were acclimatized for 7 days to housing in single cages (Makrolon type III, Indulab, Italy). Wt and HIF-1α+/- mice were then randomly assigned to a normoxia or hypoxia group. For details see paragraph on "hypoxia exposure" (see Fig. 1). 24 hours after hypoxia exposure the animals were anesthetized while still under the respective hypoxic/normoxic condition. The body weight of animals was determined and soleus muscles were rapidly dissected (<5 minutes), weighed and frozen in liquid nitrogen-cooled isopentane. Muscle weighing was avoided in the hypoxia group to reduce the exposure to "normoxic air".

Muscle disuse and reloading: Muscle disuse was provoked by 7 days of hindlimb suspension before the hindlimbs were reloaded for 24 hours in normoxia or hypoxia (see Fig. 1). Appearance, behavior and activity levels of animals were regularly observed and recorded during the interventions on an analogue scale. Animals were kept at a 12:12 light: dark cycle with water and standard chow *ad libitum*. Start time of the experiments was held constant to permit constant interval between nocturnal activity cycle and muscle harvesting.

Hypoxia exposure: The cages of singly housed mice were rapidly flushed with a mixture of nitrogen and oxygen under application of a continuous pump system (AltiTrainer200, SMTEC, Switzerland). Oxygen and carbon dioxide content was monitored online (Servomex Oxygen Analyzer 570 A; Zurich, Switzerland) and set to a PiO_2 of 70 mmHg for the hypoxia group, i.e. 10.5% O_2 ad 23°C @ 560 meters above sea level (21). This corresponds to an O_2 concentration at 5360 meters above sea level. Control animals were exposed to a PiO_2 of 138 mmHg, i.e. 20.9% O_2 ad 23°C @ 560 meters above sea level. Animals were observed twice to monitor the level of spontaneous activity and health status during the hypoxia exposure. Differences in body and soleus weight were statistically verified with a one-way ANOVA and an ANOVA with repeated measurements, respectively, with a post-

hoc test for highest significance difference (HSD; STATISTICA 6.1, StatSoft Inc, www.statsoft.com).

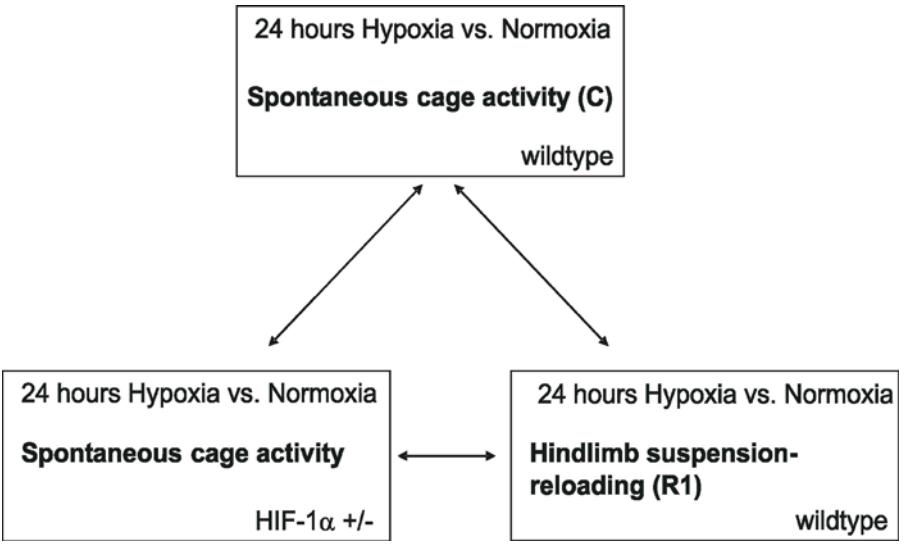

Figure 1. Experimental setup. Arrows separate between the different experimental groups.

Microarray analysis: Microarray analysis with custom-designed low-density AtlasTM cDNA expression arrays (BD Biosciences AG, Allschwil, Switzerland) for 222 mouse cDNAs associated with skeletal muscle form and function was carried out as described (6). In brief, total RNA was isolated and reverse-transcribed in parallel into labelled cDNA with 222 mRNA-specific primers and ribosomal 18S/28S rRNA using Superscript II (Life Technologies AG, Basel, Switzerland). mRNA-specific and 1:900 diluted 18S/28S cDNAs were mixed, hybridized to nylon arrays and signals of the washed filters were detected after 5 days of exposure with a phosphoImager (Molecular Dynamics, Sunnyvale, CA, USA). Batches of 4 samples were always processed simultaneously with 6 biological replica for each treatment. Data sets have been deposited at Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) under series GSE2384 according to the minimal information on gene expression (Brazma MIAME).

Statistical analyses for differential mRNA expression: Testing for transcript level differences between two experimental groups was carried out as described (43). In brief, 141 mRNAs which detection level was 30% above background (on ≥4 filters) were analyzed. The significance of an mRNA level alteration was tested with permutation analyses on L1 regression models of logarithmized raw values in scatter plots for the comparison of 2 treatments under application of the sign-test (STATISTICA 6.1). To correct for multiple tests, the False Discovery Rate method introduced by Benjamini and Hochberg was applied at a two-tailed p=0.05 (12). The expression ratio for each transcript was estimated from the mean of potentiated residues (to the base of 10) from the individual scatterplots.

The effect of reloading after muscle disuse vs. spontaneous activity on hypoxia-induced gene expression was verified using 28S-related signal ratios of the two hypoxia group after normalization to the mean of the respective normoxia group. A one-way ANOVA at $p<0.05$ was used to test the combined effect of treatment on the hypoxia-response (STATISTICA 6.1).

Hierarchical cluster analysis: For the identification of major hypoxia-dependent expression patterns, background-corrected signals of the 141 detected transcripts were normalized to the signal of stably expressed mRNAs (pixel intensity between 10,000-100,000 counts). Normalized values from the hypoxia treatments were then related to the mean normalized signal of the respective normoxia group. Following, the values of 63 mRNAs which were significantly altered with 24 hours of hypoxia vs. normoxia (and/or with reloading after muscle disuse) were extracted, log-transformed, mean-centered for genes and arrays and subjected to hierarchical cluster analysis for genes and arrays using centered correlations with public Cluster and Treeview software (8).

RESULTS

The hypoxia signature of the anti-gravitational soleus muscle-Exposure of wt mice to 24-hours of severe hypoxia had a significant effect on expression of multiple genes in soleus muscle of spontaneously active mice (table 1). These adaptations were in most cases less than 2-fold. Detailed inspection revealed that these changes involved distinct upregulation of mRNA levels of glycolytic factors (ALDOA, ALDOC, GAPDH, LDH3, PFKFB1) as well as small increases of abundant mitochondrial respiration chain constituents (CYTC, COX4A, COX5B, COX6A2) and the main oxygen transporter myoglobin (MB) (see table 1). Strikingly, a down-regulation of a main factor involved in the uptake of endothelial derived lipids (LPL) and 2 members of beta-oxidation (ACADL, HADH) was observed. Conversely, the main factor of myocellular fatty acid transport (FABP3) was increased while mRNA levels of facilitative glucose transporters were not affected in spontaneously active mice after 24 hours of hypoxia exposure. With regard to the detected factors of capillary morphogenesis, transcript expression levels of factors involved in vessel dissolution (Ang-1, Ang-2, Tenascin-C), stabilization (CD31, CDH5, Ephrin B2, VEGF-R1, VEGF-R3, TSP-1) and proliferation (PAI-1, PDGFRb) were increased (44). Finally, myogenic regulatory factors including the IGF-system and myogenic transcription factors were complex affected. The body weight of mice exposed to 10.5% hypoxia, unlike normoxia, was significantly reduced (see table 2).

The HIF-1 system and the muscular hypoxia response-We analyzed the involvement of the HIF-1 system in the transduction of the hypoxia signal. Qualitative structural analysis did not indicate significant differences in RNA content, soleus or body weight between matched HIF-1α+/- and wt mice (table 2). In normoxia, soleus muscles of spontaneously active HIF-1α+/- mice demonstrated a 35%-reduced HIF-1α mRNA level in soleus muscle vs. matched wildtype animals (table 3). Several other gene transcript involving known HIF-1 targets were moderately different expressed in soleus muscles of HIF-1α+/- vs. wt mice. These mRNA level differences concerning factors involved in fatty acid metabolism, myogenic regulation replication, capillary dissolution and hypoxia signaling

Table 1. Summary of hypoxia-induced mRNA level alterations. Hypoxia (Hyp) to normoxia (Nor) ratio of transcript levels in mouse soleus muscle ordered per gene ontology for wildtype (wt) and HIF-1α+/- cage controls, and wt mice after reloading from previous disuse. Known HIF-1 regulated genes are printed in white on black background. Transcripts which are subject to opposite regulation in wt versus HIF-1α+/- mice are highlighted in grey. Significant differences are underlined and printed in bold.

				Cage control Hyp/Nor				Reloaded Hyp/Nor	
				wt		HIF-1α+/-		wt	
Category		Gene	GenBank ID	p value	fold change	p value	fold change	p value	fold change
Metabolism	CHO metabolism	GLUT1	M23384		0.96	1.4E-03	**1.01**		1.04
		GLUT2	X16986		1.09	1.0E-07	**1.51**	5.5E-06	**1.37**
		GLUT3	M75135		0.96		n.d.	1.4E-03	**1.05**
		GLUT4	M23383	9.2E-09	**0.80**		1.00		1.04
	Glycolysis	ALDOA	Y00516	1.2E-04	**1.20**		0.96	8.7E-03	**1.23**
		ALDOC	S72537	4.4E-04	**1.29**		1.19	1.5E-11	**2.80**
		ENO2	X52380	8.6E-07	**0.82**		0.94		0.88
		GAPDH	M32599	2.8E-05	**1.21**		0.97	1.4E-03	**1.16**
		HK1	J05277		n.d.		n.d.	1.4E-03	**0.90**
		LDH1	U13687		0.90	2.8E-05	**0.87**	3.7E-03	**0.83**
		LDH3	M17587	8.7E-03	**1.14**		0.88	1.2E-04	**1.18**
		PFKFB1	X98848	1.8E-02	**1.19**		0.96	4.4E-04	**1.35**
	FA mobilization	LDLR	Z19521		0.97		n.d.	2.8E-05	**0.84**
		VLDLR	L33417		0.95	9.2E-09	**1.25**	3.7E-03	**0.84**
		FAT/CD36	L23108		1.23	1.0E-07	**1.66**	1.0E-07	**1.45**
		Scarb2	AB008553		0.97		0.93	1.0E-07	**0.75**
		LPL	M60847	9.2E-09	**0.84**	1.8E-02	**1.09**	1.4E-03	**0.78**
	FA transport	FABP4	K02109		0.95	9.2E-09	**1.49**		1.12
		FABP3	X14961	2.8E-05	**1.36**	3.7E-03	**1.28**	1.2E-04	**1.32**
	krebs cycle	PDHA1	M76727	4.4E-04	**0.85**	9.2E-09	**1.34**		0.86
	beta oxidation	ACADL	U21489	4.4E-04	**0.80**	9.2E-09	**1.23**		0.90
		HADH	D29639	5.5E-06	**0.76**		1.06		0.88
	O_2 storage	MB	X04405	2.8E-05	**1.18**		0.96	1.0E-07	**1.28**
		HO-1	M33203	2.8E-05	**1.17**	1.4E-03	**1.17**	1.2E-04	**0.66**
	respiration	CYCT	X55771	3.7E-03	**1.13**		n.d.		0.95
		COX4A	X54691	1.8E-02	**1.05**		1.05		1.07
		COX5B	X53157	4.4E-04	**1.16**		1.03	8.6E-07	**1.35**
		COX6A2	U08439	9.2E-09	**1.21**		0.90	1.0E-07	**1.33**
myogenic regulation	cell cycle	MEF2A	U30823	3.7E-03	**0.78**		0.98	1.2E-04	**0.86**
		MEF2C	L13171	1.0E-07	**0.68**	3.7E-03	**1.10**	1.0E-07	**0.72**
		MYOD	M84918		1.12		0.76	3.7E-03	**0.66**
		SRF	NM020493		0.90		0.99	8.7E-03	**1.03**
		CCND1	S78355	9.2E-09	**0.81**		n.d.	0.0E+00	**0.61**
		CDK4	L01640	1.4E-03	**1.18**		n.d.		0.84
	hormonal	GHR	M33324	4.4E-04	**0.86**		0.89		**1.02**
		IGF-I	X04480		1.34		0.63	1.4E-03	**1.82**
		MGF	NM010512		1.22		0.65	1.4E-03	**1.61**
		IGF2R	U04710		0.99		0.97	9.2E-09	**0.74**
		IGFBP4	X81582	1.0E-07	**0.85**		1.00	2.8E-05	**0.88**
		IGFBP5	L12447	0.0E+00	**0.54**	1.0E-07	**0.85**	0.0E+00	**0.66**
		IGFBP6	X81584	4.4E-04	**1.44**		1.01	8.6E-07	**1.76**
	dissolution	ADAM 2	U16242	3.7E-03	**0.80**		0.99		0.89
		Ang-1	U22516	4.4E-04	**1.64**		1.08		1.14
		Angpt-2	AF004326		1.33		1.16	8.6E-07	**2.00**
capillary remodeling		Ang-2	U22519	1.4E-03	**1.42**		1.00	1.4E-03	**1.59**
		MMP-10	Y13185		n.d.		n.d.	4.4E-04	**0.69**
		Tenascin-C	D90343	8.7E-03	**1.31**		0.88		1.25
	proliferation	ACE	J04946		1.11	8.6E-07	**1.23**	3.7E-03	**0.88**
		CD44	M27129		n.d.		n.d.	2.8E-05	**0.69**
		EGF-R	X78987	5.5E-06	**0.88**		1.04		1.02
		iNOS 2	M87039		1.31		n.d.	1.4E-03	**1.37**
		PAI-1	M33960	8.7E-03	**1.41**	1.4E-03	**0.84**		1.31
		PDGFRb	X04367	2.8E-05	**1.11**		0.97	5.5E-06	**0.88**
		MMP-14	X83536		0.95		1.03	1.4E-03	**0.89**
		Tie-2	S67051		1.00		n.d.	8.7E-03	**1.08**
		u-PA	X02389		n.d.	1.4E-03	**1.15**	1.4E-03	**1.34**
		VEGF	M95200		0.97	5.5E-06	**0.84**		1.00
		VEGF-B	U43836		0.99	1.8E-02	**1.06**		1.03
		VEGF-R2	X70842		0.95	4.4E-04	**0.92**		1.02
	stabilisation /anti-angiogenic	CD31/PECAM	L06039	8.7E-03	**1.68**		0.86	5.5E-06	**1.83**
		CDH5	X83930	1.8E-02	**1.21**		1.14	4.4E-04	**1.22**
		Ephrin B2	L25890	3.7E-03	**1.12**		0.97		0.98
		lama 4	Y09827		1.05	1.2E-04	**0.86**	5.5E-06	**1.11**
		TIMP-1	X04684	4.4E-04	**0.88**		0.94	8.6E-07	**0.76**
		TIMP-2	X62622	3.7E-03	**0.90**	1.2E-04	**1.17**		0.89
		TIMP-3	L19622		0.98	2.8E-05	**1.33**	1.4E-03	**0.78**
		TSP1	M62470	1.4E-03	**1.21**		0.88	1.4E-03	**1.22**
		VEGF-R1	X78568	3.7E-03	**1.16**		n.d.		0.99
		VEGF-R3	L07296	1.8E-02	**1.42**		n.d.	5.5E-06	**1.66**

were small but systematically affected.

Hierarchical cluster analysis of the differentially expressed mRNAs revealed that these HIF-1α heterozygous mice demonstrate systematic shifts of the hypoxia-induced gene expression patterns during spontaneous cage activity (Fig. 2). For instance the hypoxia-induced transcript levels in wt mice of gene ontologies involved in glycolysis and mitochondrial respiration were blunted (see table 1 and Fig. 3). Conversely, the down-regulated muscular mRNA levels for factors involved in fatty acid mobilization, except FABP3, were now enhanced with 24 hours of severe hypoxia in the HIF-1α+/- mice. In hypoxia, the mRNA response of several angiogenic factors and most myogenic regulators was reduced and some were oppositely affected in HIF-1α+/- vs. wt littermates.

Interaction of hypoxia with mechanical stimuli-We next evaluated the interaction of the hypoxia stress with mechanical stimuli in mice by reloading after muscle disuse. The hypoxia-induced transcript level alterations were significantly different between reloading and spontaneous cage activity ($p<0.05$; one way ANOVA). This divergence was substantiated by cluster analysis demonstrated that the hypoxia transcript signature differentiated between the two experimental groups (Fig. 2). This cluster analysis also revealed that components of the hypoxia-induced transcript signature were preserved and expanded in disused soleus muscles after 24 hours of reloading in hypoxia (Fig. 2). Interestingly, from the 50 transcripts which were significantly altered upon hypoxia, 24 follow the same expression pattern upon reloading in hypoxia. Detailed inspection revealed that the amplitude rather than the involvement of most transcripts, hypoxia-modulated level adjustments of gene ontologies involved in glycolysis, mitochondrial respiration, and capillary remodeling were reduced with reloading of disused soleus muscle compared to spontaneous cage activity (Table 1). Conversely, hypoxia-induced transcript level alterations of myogenic regulators IGF-I, MGF and IGF-BP-6 were more pronounced during reloading.

Table 2. Alterations in RNA content, soleus and body weight with 24 hours of normoxia and hypoxia.

		Normoxia		*Hypoxia*	
		total	***Δ24h***	***total***	***Δ24h***
Cage control	*RNA*	3.3 ± 0.5 μg		3.3 ± 0.3 μg	
	soleus	7.3 ± 0.2 mg *		na	
	body	22.6 ± 0.5 gr *	+0.1	20.9 ± 0.9 gr	-1.8 †
Hindlimb suspension-reloading	*RNA*	2.0 ± 0.3 μg		1.8 ± 0.3 μg	
	soleus	5.5 ± 0.4 mg		na	
	body	20.45± 0.4 gr	+0.2	20.0 ± 0.6 gr	-1.2 †
HIF-1α+/-	*RNA*	4.0 ± 0.6 μg		3.5 ± 0.5 μg	
	soleus	7.8 ± 0.4 mg *		na	
	body	22.4 ± 0.9 gr	0.0	20.2 ± 0.7 gr	-2.5 †

Values represent the mean ± SE after the 24 hour interventions (n=6). Δ denotes the mean difference values after the 24 hours of intervention. na, not assessed *, $p< 0.05$ vs. disuse-reloading in normoxia. †, $p< 0.01$ vs. values before exposure to hypoxia (ANOVA, HSD post hoc test).

DISCUSSION

Systemic hypoxia is a main stimulus of muscle phenotypical adaptations. The addition of hypoxia is known to pronounce the effects of enhanced contractile activity and muscle loading on vessel growth, protein synthesis and metabolic pathways (1, 18, 29). This hypoxia-promoted activation of biological processes in skeletal muscle may be the direct consequence of the enhanced induction of signaling cascades by local reduction of oxygen supply in muscle or mediated by central effects of hypoxia (i.e. on blood flow) (1). Thus, we reasoned that exposure of mice to a hypoxic environment for 24 hours results in a specific muscular transcript "signature" that relates to the known effects of hypoxia and which would prevail with reloading after muscle disuse. 24 hours of hypoxia was selected to reveal the sustained rather than early, and possibly muscle tissue-specific expressional adaptations to this stimulus (30, 31). The assessment of alterations of selected muscular mRNAs was carried out with validated microarray filters and accepted statistical tests (6, 43). In order to study the interaction of hypoxia with contractile activity/muscle loading, we focused on the soleus muscle in mice. This muscle's homeostasis is particularly mechano-dependent and undergoes severe atrophy and a pronounced metabolic shift with hindlimb suspension (6). This model of muscle disuse and a transgenic model for HIF-1α deficiency were thus used to pinpoint at the role of muscle activity and the HIF-1 hypoxia sensor in the muscular response to severe systemic hypoxia.

Our microarray study identifies that the hypoxia response involves distinct gene expressional adaptations that discriminates between experimental treatments but shows that basic features were preserved during reloading after muscle disuse (Fig. 2). The recorded molecular adjustments (see below) must seen in context of previous observations on muscle structural adjustments to hypoxia and resemble recent molecular observations on the response of cultured endothelial cells to hypoxia (31) and HIF-1α conditional knock-out mice to running exercise (25). In combination with the blunted RNA response to hypoxia in HIF-1α+/- mice, our molecular observations imply a key role of the hypoxia sensor HIF-1α and muscle use in the response of muscle tissue to the assumed local hypoxia.

Limitations–This is the first systematic study analyzing the broad expressional response of muscle-relevant factors to a non-lethal hypoxia stimulus. Due to the exploratory nature of this mRNA study, several considerations apply with regard to potential limitations of our conclusions. These concerns relate to the cellular origin and the translation of the observed hypoxia-induced transcript level adaptations at the protein level. Missing biochemical data on HIF-1α protein accumulation cast some uncertainty with regard to the suspected direct role of the HIF-1α subunit in the sensing mechanism that governs hypoxia-induced 0transcript expression in soleus muscle. Thus we can not rule out that the altered hypoxia response of transcript levels in HIF-1α+/- vs. wt animals is a consequence of alternative hypoxia-sensing pathways in muscle or nerve-dependent hypoxia reflexes of blood supply lines (see 1, 13).

The significant drop in body weight after 24 hours of severe hypoxia indicates that our results must be seen with regard to systemic events related to wasting (see table 2). Despite this, only a few transcript level alterations point in the similar direction as seen previously with food deprivation in the gastrocnemius muscle of mice (22), i.e. IGFBP-5, ENO1 and CD36. In fact, the majority of mRNA level alterations in mouse soleus such as those

Table 3. mRNA level differences in normoxia between HIF-1α+/- and wt mice. Significant alterations at p<0.05 as determined from microarray experiments (n=6) are indicated with an asterisk. Δ% refers to the mean percentage change (HIF-1α+/-vs.wt) of the corresponding transcript group *, determined with RT-PCR (HIF-1α+/-, n=4 vs. wt n=3).

ontology	*general trend*	*genes*	*genbank*
CHO uptake	↑Δ+*20%*	GLUT2	X16986
		GLUT3	M75135
glycolysis	↑Δ+*60%*	ALDOA	Y00516
		ALDOC	S72537
		PFKFB1	X98848
	↓Δ-*28%*	ENO2	X52380
		LDH1	U13687
		LDH3	M17587
fatty acid metabolism	↑Δ+*39%*	FAT/CD36	L23108
		VLDLR	L33417
		FABP4	K02109
		HADH	D29639
		PDHA1	M76727
	↓	Scarb2	AB008553
mitochondrial respiration	↓Δ-*10%*	COX4A	X54691
		COX6A2	U08439
	↑	COX VIIIa	U37721
myogenic regulation	↑Δ+*69%*	GHR	M33324
		IGF-I	X04480
		IGF-II	M14951
		IGFBP6	X81584
	↓Δ-*13%*	IGF2R	U04710
		CCND1	S78355
replication	↓Δ-*20%*	Pola	D17384
		Pole2	AF036898
		Top1	D10061
capillary remodeling dissolution	↑Δ+*62%*	ADAM 2 (↓)	U16242
		Ang-1	U22516
		Angpt-2	AF004326
		Ang-2	U22519
proliferation		Tenascin-C	D90343
	↑Δ+*46%*	EGF-R	X78987
		FGF R2	M86441
		PAI-1	M33960
		VEGF-R2	X70842
	↓Δ-*22%*	Itgav	U14135
		iNOS 2	M87039
		MMP-14	X83536
		PDGFRb	X04367
		PDGFb	M84453
		PDGFRa	M57683
		Tie-2	S67051
stabilisation	↑Δ+*22%*	CDH5	X83930
		Ephrin B1	U12983
		Ephrin B2	L25890
	↓Δ-*20%*	Lama 5	U37501
		Tsp2	L07803
		VEGF-C	U73620
hypoxia signaling	↓Δ-*20%*	HIF-1α *	U59496
		HIF-1β	U14333

related to glycolytic and oxidative metabolism were different to the ones observed in starved gastrocnemius. This implies that our molecular observations in hypoxia do not correlate with starvation. Finally, the uncertainty of the level of muscle use during spontaneous cage activity in hypoxia vs. normoxia in our experiments and the particular tonic, oxidative phenotype of the muscle under study argue for caution in the extrapolation of identified relationships to the muscular hypoxia response in other models and species. Nevertheless, the "molecular features" of the hypoxia response of soleus muscle as exposed by our microarray exploration allow to hypothesize on the interaction of muscle use with the HIF-1α-mediated muscular hypoxia response.

The pattern of hypoxia-induced transcript level alterations-The contention of a specific molecular hypoxia "signature" is indicated by our findings showing a preservation of this signature for transcript levels in the soleus muscle in mice after disuse atrophy (see Fig. 2). This is suggestive for hypoxia to represent an important stimulus setting the direction of the muscular gene responses. The molecular hypoxia signature involved enhanced gene expression of capillary remodeling factors, opposite mRNA adaptations of oxidative, glycolytic and lipidic metabolic pathways and modified expression of myogenic regulators (see Fig. 4). These observations thus extend the historical assumptions on enhanced mitochondrial biogenesis and the increased glycolytic energy production in hypoxia to the mechanistic level. At the same time, this adds novel evidence for alterations of fatty acid transport and enhanced remodeling of the capillary network (Fig. 3).

Particularly, the co-incident upregulation of factors involved in distinct metabolic pathways indicates expressional reprogramming of distinct energy metabolic processes in hypoxia-exposed soleus muscle. The increased mRNA levels in glycolytic factors thereby reflect those enzymatic adaptations as previously noted in gastrocnemius muscle (33). This contention on a comprehensive shift from fatty acid to carbohydrate consumption is supported by the down-regulation of the verified factors involved in uptake of lipids from the vasculature into muscular tissues, i.e. VLDLR, LDLR and LPL, and mitochondrial beta oxidation, i.e. ACADL and HADH, in the soleus muscle with reloading in hypoxia. In this regard, the increased mRNA level of respiration factors is puzzling as this suggests enhanced turnover or accumulation of constituents of the mitochondrial oxidative pathway in hypoxia.

HIF-1α and the hypoxic response in soleus muscle-The setting of ambient air to 10.5% O_2 represents a severe insult for the exposed mice. A rapid drop in muscular oxygen tension due to this lowering of FiO_2 to a simulated altitude of 5360 meter above sea level is indicated from recent studies (28, 32, 41, 39). Evidence for global tissue hypoxia in our study was provided by the visual de-oxygenation of animals as seen due to the shift form a red to a bluish body color within minutes of exposure to 10.5% O_2. Consequently, important hypoxia-dependent processes may be initiated in muscle tissues.

In various organs HIF-1α protein is known to be stabilized with a specific time-course in response to a lowering of FiO_2 to 6% (40). Our western blot analysis with several antibodies failed to detect a suspected accumulation of stabilized HIF-1α protein in whole cell lysates of the soleus muscle during the time course of the 24 hour response in 10.5% (and 6%) O_2 (data not shown). Subsequent experiments with more specific antisera and nuclear fractions are now necessary to provide the ultimate biochemical proof for a direct muscular involvement of HIF-1α protein in the observed muscular transcript response in hypoxia. The blunted hypoxia response of multiple transcripts in the HIF-1α+/- animals with a 35%-

reduced basal level of HIF-1α mRNA therefore were the confounding observation of our study. This indeed points towards an active role of HIF-1α in the mRNA response of hypoxic muscle (see Fig. 4). The notion of a master role of HIF-1α in hypoxia-induced gene expression in muscle is corroborated with the similitude of biological processes which response to endurance exercise is blunted in conditional-knock-out HIF-1α animals demonstrating a 40% reduction of the HIF-1α allele (25).

In this regard, the up-regulation of transcripts for factors involved in vascular lipid uptake and mitochondrial beta oxidation in HIF-1α+/- animals was a surprising observation because these concentrations were reduced upon hypoxia exposure in wild type mice. This invokes the existence of HIF-1α dependent negative feedback mechanism which controls transcript synthesis or degradation of the former mRNAs. Decreased oxygenation initiates a variety of signals that lead to the growth of blood vessels (1). The blunted response of remodeling factors therefore provides evidence that the suspected remodeling response of the endothelium is mediated by HIF-1-dependent hypoxia sensing.

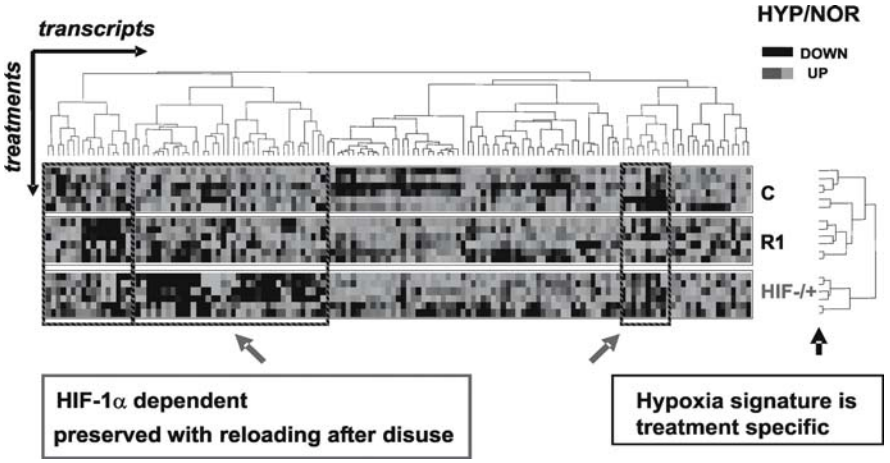

Figure 2. Muscular hypoxia expression "signature". Graph depicts that the muscular hypoxia-induced gene expression patterns distinguish between experimental groups. Hypoxia vs. normoxia expression ratios of hypoxia-modified transcripts in soleus muscle from wildtype mice during spontaneous cage activity (C) or one day of reloading of after disuse (R1) or HIF-1α+/- mice was analyzed by hierarchical cluster analysis. Clusters of mRNA levels with a particular level of co-regulation are demarcated.

Interaction with muscle use-Our findings showing a moderately reduced but expanded, muscular hypoxia signature of transcript levels in reloaded animals after disuse atrophy extend the evidence for a possible interference of muscle use and hypoxia with regard to muscular adjustments to the molecular level (reviewed by 11, 29). Meanwhile meta analysis demonstrated a certain overlap in the gene ontologies for HIF-1α-dependent muscular transcript adaptations to hypoxia in our study and in the previous report on endurance exercise in HIF-1α-dependent mice (25). This possibly relates to the lowering of the muscular HIF-1α mRNA level increase with reloading in hypoxia (minus 20%) or a lower uptake of contractile activity in disused than normal skeletal muscle.

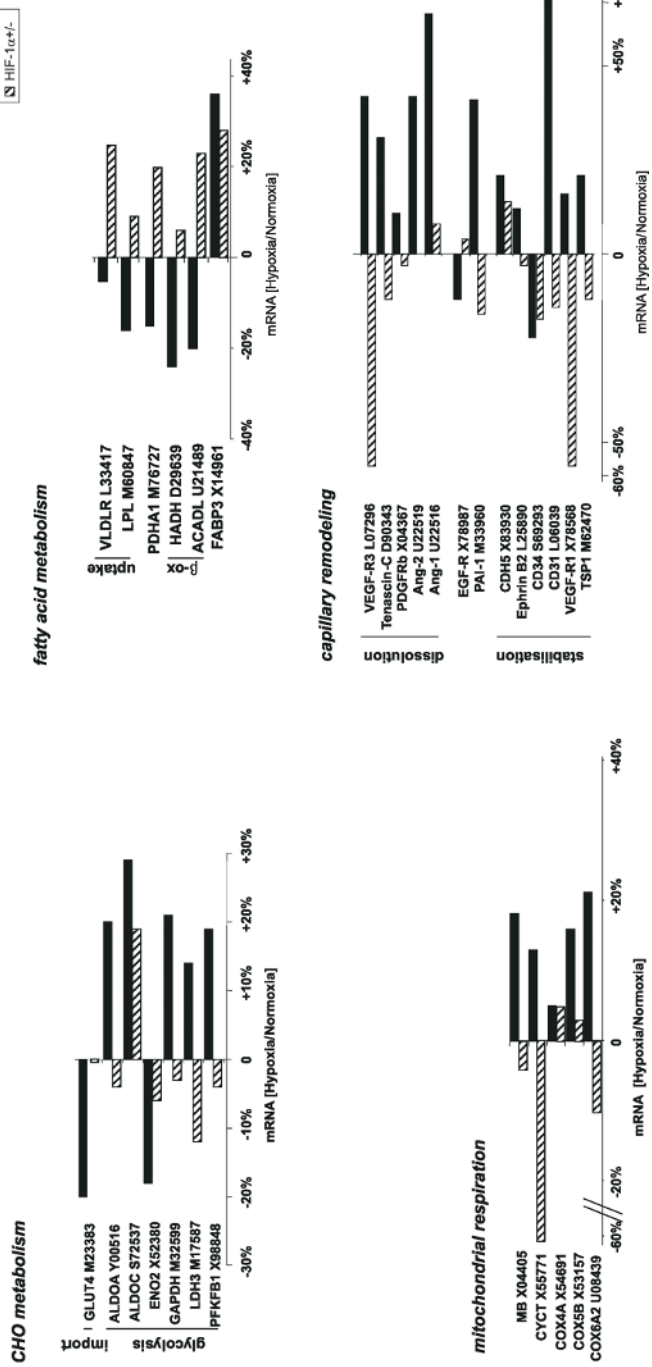

Figure 3. Hypoxia-induced alterations and HIF-1α-dependence of transcripts within individual gene ontologies

In this regard, the lower amplitude of hypoxia-induced mRNA alterations in disused soleus muscle may reflect reduced HIF-1-mediated oxygen sensing due to reduced potential for stabilization of HIF-1α signaling molecules during reloading in hypoxia (see Fig. 4). On the other hand, animal monitoring indicated an initial drop in the spontaneous physical activity of mice upon exposure to 10.5% hypoxia.

In parallel, reloading of disused muscle provides a relative high increase in muscle loading vs. the load changes seen with spontaneous activity in the cage controls. This "predominance" of mechano-dependent events is also manifested by the additional upregulation of expression levels for the mechano-sensitive insulin-like growth factor I (IGF-I) transcripts and the related MGF splice form and IGFBP6 after reloading of disused muscle in hypoxia (see Fig. 2 and table 1; 10). The novel observation of hypoxia-induced expressional regulation of the IGF-I system therefore is seen to be related to the reported effect of hypoxia on protein synthesis (reviewed in 10, 18, 29). Our data therefore indicate that the muscle hypoxia response is part of a potential part of an auto-/paracrine loop involved in the anabolic effects of hypoxia exposure (10, 18). Overall our observations provide a molecular counterpart for the structural observations that muscle activity is an important co-variable of hypoxia-induced alterations in muscle fiber phenotype (4, 26).

The (sub)cellular mechanism of the hypoxia response-The measure of mRNA level alterations in a homogenate begs the question as on the cellular origin and functional implications of the gene expressional response. In this regard, the expression of several of the quantified transcripts is confined to one particular cell type. Consequently, these level changes are indicative for activation of distinct cellular populations in muscle by 24 hours of severe hypoxia. For instance, several of the altered mRNA levels of factors involved in capillary remodeling are preferentially/uniquely expressed in the endothelial cell type (CD31, CDH5, Ephrin B2). Similarly, the match in several mRNA level adaptations of soleus muscle with the ones seen in hypoxic endothelial cultures (see table 3) suggests a major contribution of endothelial cells to the muscular transcript alterations after 24 hours of hypoxia in our study (30, 31). Likewise, muscle fibers represent the major source of glycolytic and mitochondrial respiratory factors (5, 34).

Concerning the transcript level alterations for endothelial-specific factors, evidence for a distinct kind of capillary remodeling becomes manifest. For instance, the increase in mRNAs involved in vessel dissolution (Ang-1, Ang-2, Tenascin-C, VEGF-R3, PDGFRb) and stabilization (CD31, Ephrin B2, CDH5, VEGF-R1, TSP-1), concomitant with the absence of consistent alterations for promoters of vascular proliferation (VEGF, EGF-R, PDGFRb) supports that hypoxia *per se* is not a sufficient stimulus for capillary neo-formation in muscle (19, 26). Similar complex expressional induction of dissolution (proliferation) and stabilization factors of capillaries has been noted recently during capillary formation (23) and the hypoxic response of different vascular beds (30). Moreover, inconsistent changes in factors related to cell proliferation, i.e. CCND1, PCNA, EGF-R, mimics the anti-proliferative response seen in endothelial cells under hypoxic stress (31). The observed mRNA alterations in our study therefore may indicate proliferation-independent shape alterations of the capillary bed such as those related to intussuseptive vessel growth (1, 7, 21, 44). This is in line with the initiation of morphological alterations in capillaries (reviewed by 11) and long held speculations on the changes of vascular tortuosity in hypoxia exposed muscle (42) .

With regard to the alterations of mitochondrial transcripts two potentially important re-

lationships become apparent. First, the upregulation of myoglobin (MB), CYTC, COX4A, COX5B, COX6A2 mRNAs (Table 1, Fig. 3) represent the first evidence for an expressional regulation of respiratory chain constituents in muscle upon severe hypoxia alone. This observation is compatible with the initially supported but later refused contention of Reynafarie that hypoxia alone may increase mitochondrial performance (see 4, 19). Secondly, the opposite transcript level alterations of factors involved in beta-oxidation provides evidence for a differential regulation of the expressional makeup of mitochondria. We do not know whether these transcript responses necessarily translate into corresponding change of mitochondrial protein composition. Future studies now need to test whether the hypoxia stimulus alone is sufficient to promote selective turnover or compositional alterations of mitochondrial content.

Outlook-Muscle disuse and hypoxia take important part in the onset of clinical syndromes such as chronic obstructive pulmonary disease, chronic heart failure and peripheral arterial occlusive disease (9). For instance, deconditioned muscles of chronic obstructive pulmonary disease (COPD) patients experience frequent episodes of tissue hypoxia during rehabilitation (3, 24). The peripheral muscle abnormalities observed with these syndromes include a shift from oxidative to glycolytic energy metabolism and a limitation of blood flow via a reduction of vasculature supply (reviewed in 9, 27, 35). These adaptations are of striking similarity to the adjustments seen with muscle disuse and hypoxia in the hindlimb suspension model. The molecular observations on the transcriptome response to hypoxia in disused muscle therefore are of potential relevance for the understanding of the role of ischemia in deconditioned human muscle tissues. This notion is supported by the fact that in accordance with our study, cytochrome oxidase activity is paradoxically increased in skeletal muscle of COPD patients when the mitochondrial machinery involved in the generation of reduction equivalents from organic C2 substrates was reduced (2, 37).

CONCLUSION

The present microarray study demonstrates that 24 hours of severe hypoxia provokes a specific gene expressional response in anti-gravitational mouse soleus muscle via the hypoxia sensor HIF-1α and interacts with mechanical signals as modified by the level of muscle use. The mapped transcript "signature" implicates a scenario for distinct expressional mechanisms to be involved in the early muscular adaptations to severe hypoxia. For most, these mRNA level adjustments resemble the response of endothelial cells to severe hypoxia and link to the reported metabolic consequences of chronic hypoxia and muscle use on skeletal muscle makeup. This molecular picture suggests that short term severe hypoxia promotes capillary remodeling via an activation of the endothelial cell population and supports a shift toward an enhanced potential for glycolysis and mitochondrial respiration with a concomitant down-regulation of vascular import and beta oxidation of fatty acids.

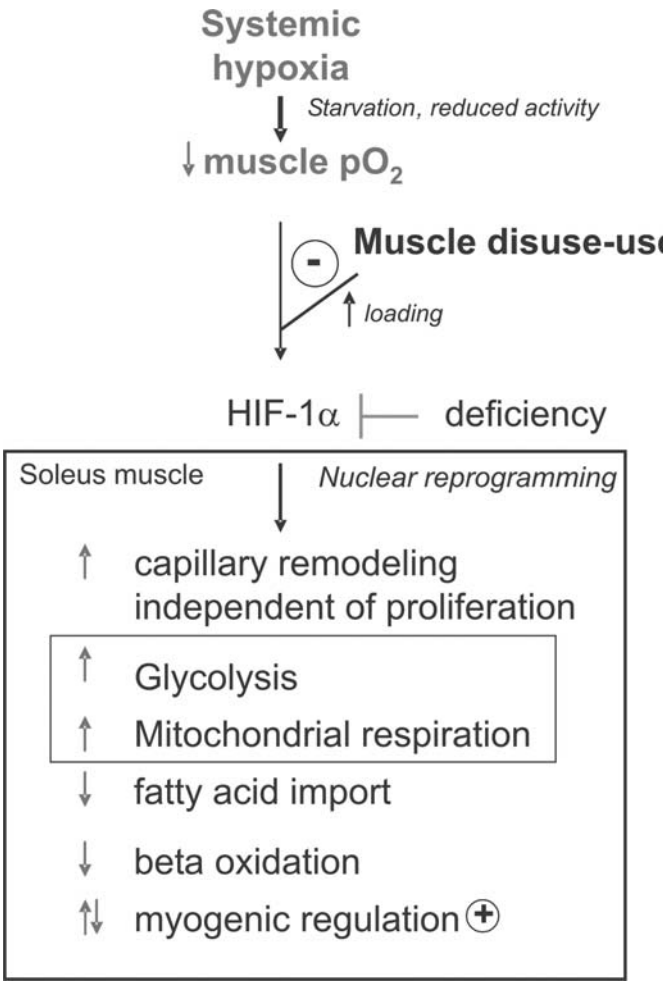

Figure 4. Synopsis and proposed model of hypoxia signal integration. Systemic hypoxia provokes nuclear programming of different gene ontologies (boxed) in soleus muscle via a drop in muscle oxygen tension (piO_2) and the activation of the hypoxia sensor HIF-1α. Consequently, this hypoxia response is blunted in HIF-1α heterozygous deficient mice. Other potential co-stimuli involved in the muscular response during systemic hypoxia are indicated in italics. Enhanced muscle activity such as with reloading after muscle disuse reduces the amplitude of hypoxia-induced transcript level alterations via enhanced muscle loading (except for myogenic factors whose transcript response is enhanced).

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ABBREVIATIONS

ACADL, Acyl-CoA dehydrogenase; ALDOA, Aldolase A; ALDOC, Aldolase C; Ang-1, Angiogenin; Ang-2, Angiogenin-2; CCND1, cyclin D1; CD31, Platelet endothelial cell adhesion molecule precursor; CDH5, Vascular endothelial-cadherin precursor; CYTC, Cytochrome C; COX4A, Cytochrome C oxidase subunit 4A; COX5B, Cytochrome C oxidase subunit 5B; COX6A2, Cytochrome C oxidase subunit 6A2; EGF-R, Epidermal growth factor receptor; Ephrin B2, Ephrin type-B receptor 2, GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HADH, Short chain 3-hydroxyacyl-CoA dehydrogenase; HIF-1α, hypoxia-inducible factor-1 alpha;HIF-1α, hypoxia-inducible factor-1 beta LDH3,lactate dehydrogenase 3; LDLR, low-density lipoprotein receptor; LPL, Lipoprotein lipase; FABP3, (heart) fatty acid binding protein 3; IGF-I, Insulin-like growth factor I; IGFBP6, Insulin-like growth factor binding protein 6; MB, Myoglobin; MGF, mechano-growth factor (IGF-I splice form); PAI-1, Plasminogen activator inhibitor-1; PCNA, Proliferating cell nuclear antigen; PDGFRb, Beta platelet-derived growth factor receptor; PFKFB1, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1; TSP-1, Thrombospondin 1; VEGF, Vascular endothelial growth factor; VEGF-R1, Vascular endothelial growth factor receptor 1; VEGF-R3, Vascular endothelial growth factor receptor 3; VLDLR, Very low-density lipoprotein receptor

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Chapter 17

ROLE OF THE RED BLOOD CELL IN NITRIC OXIDE HOMEOSTASIS AND HYPOXIC VASODILATION

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Abstract: Nitric oxide (NO) regulates normal vasomotor tone and modulates important homeostatic functions such as thrombosis, cellular proliferation, and adhesion molecule expression. Recent data implicate a critical function for hemoglobin and the erythrocyte in regulating the bioavailability of NO in the vascular compartment. Under normoxic conditions the erythrocytic hemoglobin scavenges NO and produces a vasopressor effect that is limited by diffusional barriers along the endothelium and in the unstirred layer around the erythrocyte. In hemolytic diseases, intravascular hemolysis releases hemoglobin from the red blood cell into plasma (decompartmentalizes the hemoglobin), which is then able to scavenge endothelial derived NO 600-fold faster than erythrocytic hemoglobin, thereby dysregulating NO homoestasis. In addition to releasing plasma hemoglobin, the red cell contains arginase which when released into plasma further dysregulates arginine metabolism. These data support the existence of a novel mechanism of human disease, hemolysis associated endothelial dysfunction, that potentially participates in the vasculopathy of iatrogenic and hereditary hemolytic conditions. In addition to providing an NO scavenging role in the physiological regulation of NO-dependent vasodilation, hemoglobin and the erythrocyte may deliver NO as the hemoglobin deoxygenates. Two mechanisms have been proposed to explain this principle: 1) Oxygen linked allosteric delivery of S-nitrosothiols from S-nitrosated hemoglobin (SNO-Hb), and 2) a nitrite reductase activity of deoxygenated hemoglobin that reduces nitrite to NO and vasodilates the human circulation along the physiological oxygen gradient. The later newly described role of hemoglobin as a nitrite reductase is discussed in the context of hypoxic vasodilation, blood flow regulation and oxygen sensing.

Key Words: SNO-Hb, hemoglobin, vasodilation, nitrite, nitric oxide, hemolysis, arginase

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INTRODUCTION

Hypoxic vasodilation is a highly conserved physiological response required to match blood flow and oxygen delivery to tissue metabolic demand. This systemic response has been appreciated for more than 100 years since the initial description by Roy and Brown in 1879 (76). Feedback hypoxic vasodilation requires oxygen or pH sensing to detect an "error signal" in the normal relationship between delivered blood oxygen content and tissue oxygen consumption (90). This error signal leads to the feedback generation of vasodilatory effectors that increase blood flow to maintain the balance of oxygen delivery and oxygen consumption. In mammals such vasodilation occurs as the hemoglobin desaturates from 60 to 40%, around a partial pressure of oxygen ranging from 40-20 mm Hg (75). Recent measurements of microcirculatory oxygen tension and hemoglobin oxygen saturation suggest that this degree of deoxygenation occurs within the resistance arterioles, especially in the case of skeletal muscle (87). In other tissues, such as heart and brain, more oxygen is extracted within the capillary network. Work by Segal and Duling suggests that NO or ATP delivery to the capillary circulation produces retrograde intracellular propagation of a vasodilating signal to the precapillary resistance vessels (80-82). These data in aggregate suggest that the oxygen or pH sensor is responsive to tissue oxygen partial pressures of 20-40 mm Hg and hemoglobin saturations of 40-60% (around the hemoglobin P_{50}).

Despite the physiological appreciation of this conserved blood flow response to hypoxia, the identity of the oxygen sensor mechanism and the specific feedback vasodilators remains uncertain and controversial. Even the site of sensing remains unknown with oxygen sensing and vasodilation either occurring within the A3-A4 arterioles or in the capillary network with retrograde propagation of a vasodilating signal through endothelium to the precapillary resistance arterioles. A number of mediators have been considered, including adenosine, nitric oxide (NO), K_{ATP} channels, endothelium derived hyperpolarizing factor (candidates include CO, H_2O_2 or $ONOO^-$), and prostacyclin, however, blockade of many of these pathways fails to completely inhibit hypoxic vasodilation (90, 91). These studies suggest that the system is either intricate and overbuilt with multiple effectors or that other undiscovered pathways exist.

An alternative paradigm has been advanced since 1995: That hemoglobin per se is the oxygen sensor with the oxygen linked allosteric structural transition of the hemoglobin tetramer from the oxygenated conformation (relaxed or R state) to the deoxygenated conformation (tense or T state) signaling the release or generation of a vasodilating signal from the erythrocyte (12). The first such hypothesis suggested that this R-to-T transition produced a release of ATP from the erythrocyte, which by binding to purinergic receptors in endothelium resulted in vasodilation (8, 12, 24, 37). This mechanism is supported by the observation of increasing concentrations of ATP in venous blood following hypoxia, and the in vitro generation of ATP from hypoxic or acidic erythrocytes. Evidence for retrograde propogation of the ATP/purinergic receptor/eNOS signal from the capillaries to precapillary arterioles further supports this hypothesis (12). The details and experimental evidence supporting this hypothesis will be covered in detail by Dr. Sprague's contribution to this series.

The second two hypotheses suggest that hemoglobin deoxygenation results in NO (equivalent) release from the red blood cell and subsequent NO-dependent vasodilation, however, the proposed mechanisms are fundamentally different. The first prosposed mech-

anism is that S-nitrosated hemoglobin (SNO-Hb) releases S-nitrosothiols during hemoglobin deoxygenation with subsequent generation of NO and vasodilation (25, 26, 38, 86). The second proposed mechanism suggests that hemoglobin is an allosterically regulated nitrite reductase which reduces nitrite to NO by deoxyheme as hemoglobin deoxygenates (5, 56). The present review will briefly review the overall role of the erythrocyte in vascular NO homeostasis, the specific mechanistic challenges facing the SNO-Hb hypothesis and then summarize the mechanism and emerging data supporting the nitrite reductase hypothesis.

NITRIC OXIDE AS A PARACRINE AND ENDOCRINE VASODILATOR (Figure 1)

Nitric oxide is produced from endothelial NO synthase and participates in the regulation of basal blood vessel tone and vascular homeostasis (antiplatelet activity, modulation of oxidant stress, endothelial and smooth muscle proliferation and adhesion molecule expression) (15, 33, 34, 64, 65). NO is a paracrine signaling molecule as it is produced in endothelium and then diffuses to vicinal smooth muscle, binds avidly to the heme of soluble guanylyl cyclase which produces cGMP, activates cGMP dependent protein kinases ultimately leading to smooth muscle relaxation. NO that diffuses into the lumen of the blood vessel is expected to react at a nearly diffusion limited rate (10^7 $M^{-1}sec^{-1}$) with both oxy- and deoxyhemoglobin to form methemoglobin/nitrate and iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO), respectively (62). These reactions limit the half life and diffusional distance of NO in blood (<2 millisecond half time) and maintain NO as a paracrine vasoregulator . The rapid irreversible nature of these NO-hemoglobin reactions and the massive concentration of hemoglobin in blood (10,000 μM heme) also suggest a great paradox in vascular biology: while we know that NO is a paracrine vasodilator, kinetic calculations suggest that all of the NO produced by endothelium should be inactivated by hemoglobin and the sphere of diffusion of NO should be extremely limited, i.e. diffusion to adjacent smooth muscle should be impossible (44).

However, we know that such diffusion is indeed possible, and the proposed solution to the paradox is illustrated in the central panel of Figure 1: during normal physiology the reaction of NO with hemoglobin is limited by compartmentalization of hemoglobin within the erythrocyte membrane (79). This compartmentalization of hemoglobin from endothelium creates two diffusional barriers: a cell free diffusion barrier along the endothelium in laminar flowing blood (3, 46) and an unstirred bulk diffusional barrier around the erythrocyte membrane (49). Additional studies suggest an intrinsic barrier within the membrane and submembrane protein matrix that further limits NO entry (29). Similar diffusional barriers modulate oxygen diffusion across the erythrocyte (4). Such barriers suggest a potential role for plasma enrichment in the microcirculation in NO homeostasis (the "Fahreus-Lindqvist" effect) and explain the morbidity and mortality of stroma-based blood substitutes and hemolytic disease (62, 73).

Hemolysis associated endothelial dysfunction and vasculopathy

Indeed, hemolytic diseases such as sickle cell disease and paroxysmal nocturnal hemo-

globinuria are associated with relative hypertension (compared to the hypotension of non-hemolytic anemias) (74), pulmonary hypertension (19, 39), esophageal and smooth muscle dystonias during paroxysms of hemolysis (28), and recently, in the case of sickle cell disease, the characterization of a state of NO resistance (73). Vasodilation during infusions of NO donors (nitroprusside, nitroglycerin, NONOates) is blunted in patients with sickle cell disease (10, 73) and in transgenic mouse models of sickle cell disease (41, 42, 60), and this resistance to NO correlates with plasma hemoglobin levels (42, 73). Not only does cell free plasma hemoglobin released during hemolysis disrupt the normal diffusional barriers but may also extravate into the extracellular space and directly intercept NO diffusing between endothelium to smooth muscle (58, 62). In addition to the release of hemoglobin during hemolysis, the red blood cell also contains large quantities of arginase, such that hemolysis increases plasma levels of this enzyme and metabolizes arginine to ornithine, reducing the substrate for endothelial NO synthase (19, 55, 83). Impairment of endothelium-dependent vasodilation leads to a state of hemolysis associated endothelial dysfunction, and with chronic hemolysis, a progressive proliferative vasculopathy. It is increasingly clear that multiple systems have evolved to limit the toxicity of cell free plasma hemoglobin, including the high molecular weight haptoglobin system (prevents extravasation of hemoglobin and limits NO scavenging) (11, 57, 92), hemopexin, CD163 hemoglobin scavenger protein (which not only mediates haptoglobin-hemoglobin clearance but also upregulates IL-10 and hemeoxygenase 1) (69), and the hemeoxygenase 1/biliverdin reductase/p21 pathways which exert anti-inflammatory, anti-oxidant and anti-proliferative effects (1, 13, 59, 63, 77).

Endocrine properties of NO

In addition to a paracrine vasodilator function, there is increasing appreciation that NO may be stabilized by the formation of NO modified proteins, peptides and lipids, as well as by oxidation to the anion nitrite. The principle that NO may be thus stabilized in blood, and the inactivation reactions with hemoglobin thus limited, was first proposed by Loscalzo and Stamler. They hypothesized that NO (abstraction of an electron required) could form a covalent bond with cysteine residues on albumin to form S-nitrosated albumin (SNO-albumin) (78, 85). This paradigm was later extended by the Stamler group to S-nitrosated hemoglobin (SNO-Hb) (38). While this field is extremely controversial, largely secondary to major questions about the concentrations and importance of SNO-albumin and SNO-hemoglobin in the human circulation (reported values range from a few μM to undetectable, with more modern methodologies documenting levels of less than 10 nM) (18, 20, 50, 70, 71, 84, 92), it is likely that there are a number of intravascular species capable of endocrine vasodilation, including S-nitrosothiols (61, 85), nitrite (5), N-nitrosamines (27, 48, 70), iron-nitrosyls (18), and recently identified nitrated lipids (47). This review will briefly review the SNO-Hb hypothesis, the major challenges to this theory, and then focus on the role of nitrite in vasoregulation and hypoxic vasodilation.

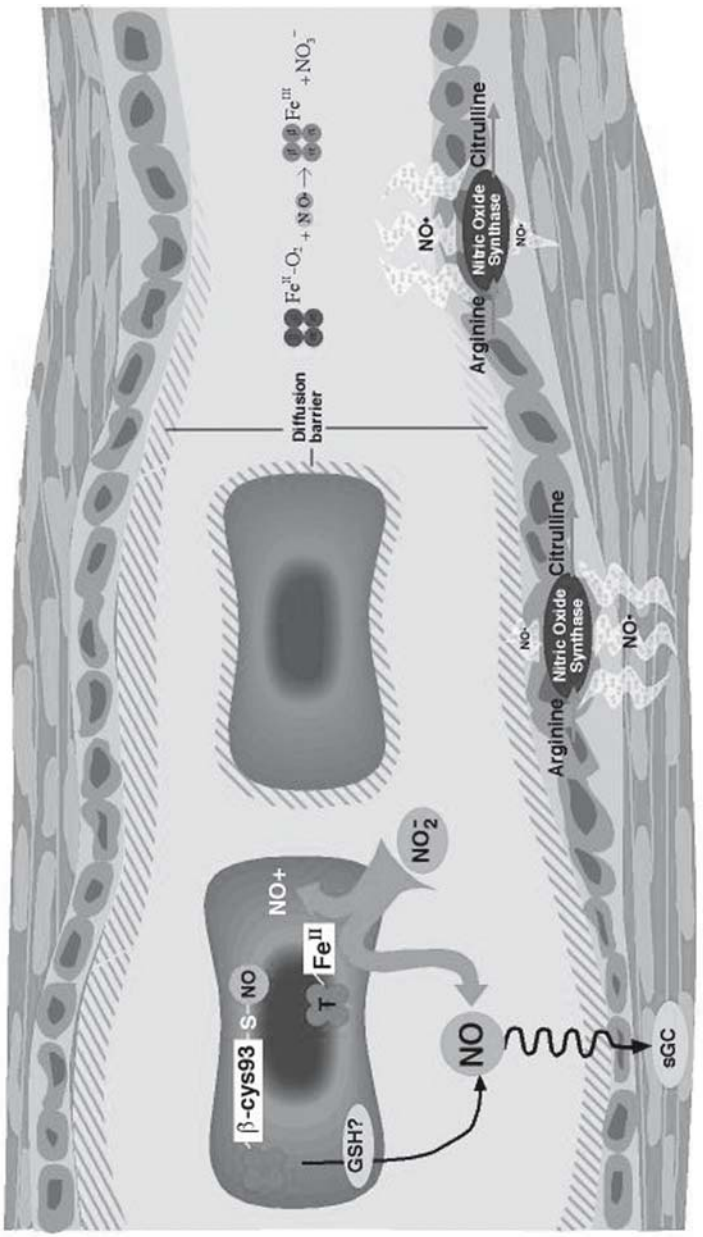

Figure 1. Endocrine, paracrine and autocrine properties of NO. Nitric oxide as a paracrine vasodilator (middle of figure), whose scavenging by hemoglobin is limited by the compartmentalization of hemoglobin within the intact erythrocyte. Nitric oxide as an endocrine vasodilator (left side of figure) being transported in blood as nitrite or an S-nitrosothiol. Nitric oxide as an autocrine vasodilator (right side of figure), whose diffusion is limited by scavenging by cell free plasma hemoglobin released during pathological hemolysis. Adapted with permission from: Schechter AN and Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med* 348: 1483-1485, 2003.

The SNO-hemoglobin hypothesis

Based on observed artery-to-vein gradients in SNO-Hb in the rat and the ability of S-nitrosated hemoglobin to vasodilate aortic ring preparations and the rat circulation it was proposed that NO represented a third gas molecule in the human respiratory cycle (26, 38, 86). A complicated mechanism was proposed suggesting that NO produced by the endothelium would react with a vacant heme on oxygenated hemoglobin (three $HbFe^{II}$-O_2 per tetramer and one $HbFe^{II}$-NO per tetramer) and thus trap and "preserve" the NO on hemoglobin (25, 52). The NO would lose an electron (mechanism not demonstrated) and then migrate to the ß-globin chain cysteine 93 residue to form an S-nitrothiol bond. This SNO-Hb would then transfer the NO+ group by transnitrosation to the erythrocyte membrane anion exchange protein (AE1 or band 3) thiols followed by export of a yet to be identified intermediate species (called X-NO) (68). This would presumably be an S-nitrosothiol which would need to be reduced to NO to activate soluble guanylate cyclase.

While the principle was elegant, the mechanism has been severely challenged (Figure 2) (17). Multiple laboratories have now shown that NO does not bind preferentially to vacant hemes on oxygenated hemoglobin (cooperative NO binding is not observed) (18, 30, 31, 40, 96). The required transfer of NO from the heme to the cysteine has not been observed using electron paramagnetic resonance spectroscopy (unless large concentrations of nitrite contaminate the experiment) (95). Importantly, this transfer requires the abstraction of an electron from the NO and a mechanism for this reduction has not been determined over the last 9 years. Finally, multiple groups have been unable to reproduce the levels of both SNO-albumin or SNO-hemoglobin reported by the Stamler group, and the artery-to-vein gradients have not been detected in the human circulation by other groups (18, 22, 50, 61, 70-72, 95). Finally, SNO-Hb is not stable in the reductive intra-erythrocytic environment at 37°C and is rapidly reduced by intracellular glutathione and ferrous heme (6, 7, 22). Thus the mechanism for formation, levels in the circulation, and oxygen dependent delivery of the S-NO group have all been challenged.

While our work suggests that SNO-Hb does not participate in the process of hypoxic vasodilation in the basal human circulation, we do find support for the principle that the red blood cell and hemoglobin participates in oxygen dependent NO homeostasis. Rather than hemoglobin being a reservoir of S-nitrosated hemoglobin, we find that hemoglobin is an enzymatic nitrite reductase with a deoxyheme-nitrite reaction generating NO as hemoglobin deoxygenates within the circulation (5, 16).

Vasoactivity of nitrite in the human circulation

While large doses of nitrite given as an antidote for cyanide poisoning clearly produces hypotension in humans (94), the large concentrations of nitrite required to vasodilate aortic ring bioassay systems led to a dismissal of nitrite as a vasoactive reservoir of NO in the circulation. Indeed, nitrite at concentrations exceeding 100 μM was shown to vasodilate aortic ring bioassays by Furchgott as far back as 1952, and shown by Murad and Ignarro to activate guanylate cyclase in the mid 1970's and early 1980's (14, 35, 36, 54). However, studies published by Laur and colleagues suggested that nitrite had no intrinsic vasodilator activity and led to a premature dismissal of nitrite as a physiological vasodilator (45, 51, 67).

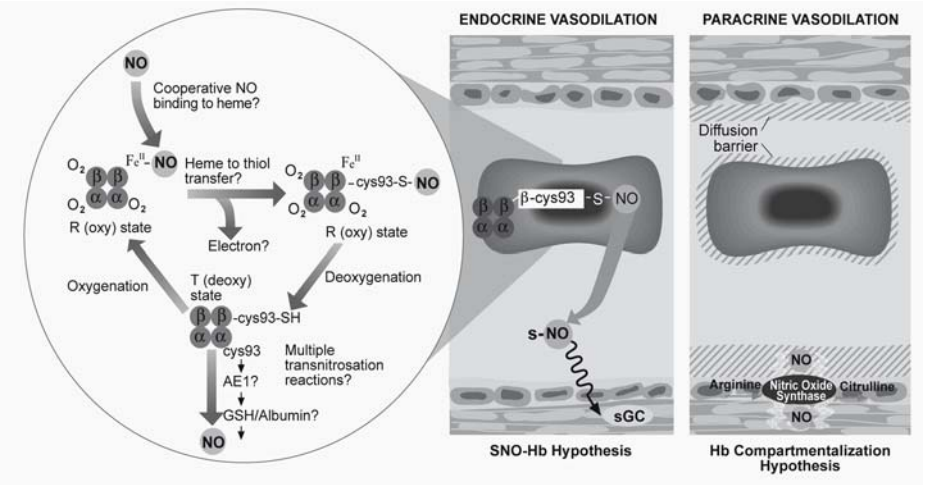

Figure 2. Mechanism proposed for SNO-hemoglobin mediated hypoxic vasodilation (left panel). A complicated mechanism was proposed suggesting that NO produced by the endothelium would react with a vacant heme on oxygenated hemoglobin (three $HbFe^{II}$-O_2 per tetramer and one $HbFe^{II}$-NO per tetramer) and thus trap and "preserve" the NO on hemoglobin (25, 52). The NO would lose an electron (mechanism not demonstrated) and then migrate to the ß-globin chain cysteine 93 residue to form an S-nitrothiol bond. This SNO-Hb would then transfer the NO+ group by transnitrosation to the erythrocyte membrane anion exchange protein (AE1 or band 3) thiols followed by export of a yet to be identified intermediate species (called X-NO) (68). This would presumably be an S-nitrosothiol which would need to be reduced to NO to activate soluble guanylate cyclase. The question marks reflect specific challenges to the mechanism discussed in the text. Figure reproduced with permission from: Gladwin MT, Lancaster JR, Freeman BA, and Schechter AN. Nitric oxide's reactions with hemoglobin: a view through the SNO-storm. *Nat Med* 9: 496-500, 2003.

Despite the apparent lack of bioactivity of nitrite in these more recent studies, we observed arterial-to-vein gradients in nitrite across the human forearm, with increased consumption of nitrite during exercise stress, suggesting that nitrite was metabolized across the peripheral circulation (21). We therefore hypothesized that nitrite might be reduced to NO during hypoxic and acidic stress by the actions of xanthine oxidoreductase (23, 53) or by acidic reduction (disproportionation) (97). To test this we infused nitrite into the forearm brachial artery of 18 healthy volunteers and to our surprise, observed substantial vasodilation, even without exercise stress. Nitrite was remarkable potent, increasing blood flow by 170% at 200 µM and by 22% at 2.5 µM. Even levels of 900 nM vasodilated during exercise stress with concurrent NO synthase inhibition with L-NMMA (Figure 3) (5). A vasodilation at these concentrations under normal physiological non-stress conditions was inconsistent with a mechanism of reduction by xanthine oxidoreductase or disproportionation, as both of these pathways require very low pH and extreme hypoxia, thus suggesting an alternative mechanism of nitrite bioactivation. Additional studies have been published in the last year confirming the vasodilating effects of nitrite (32, 43, 88, 89, 93).

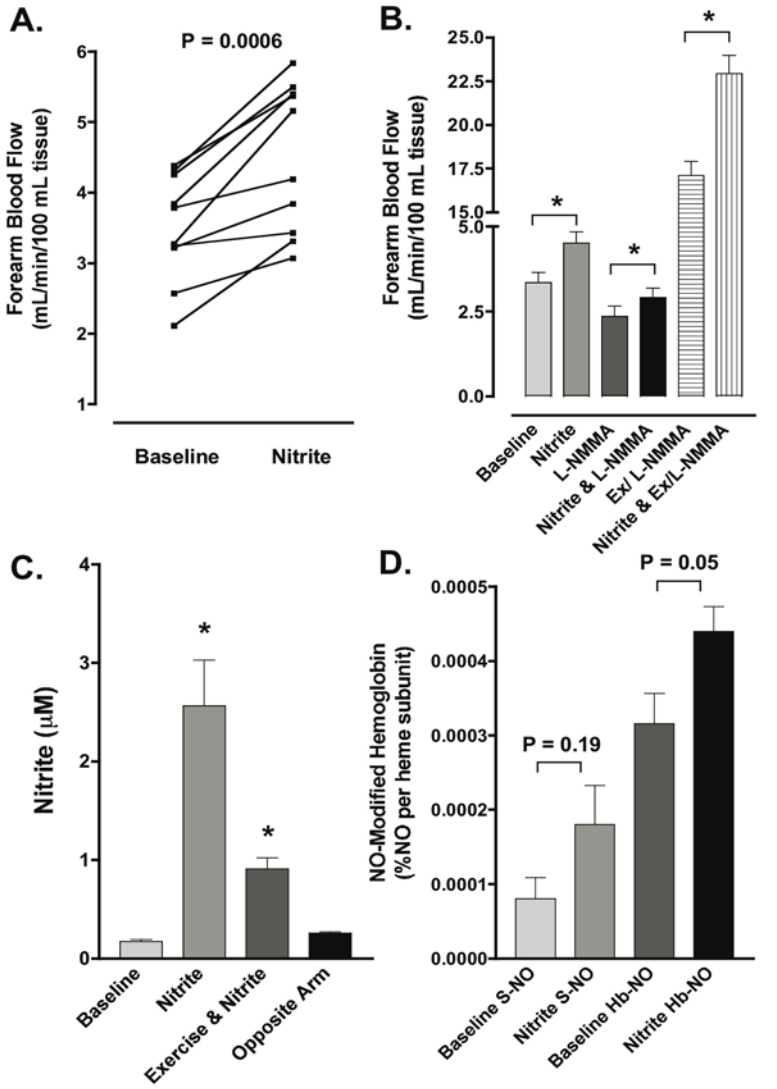

Figure 3. Nitrite vasodilates the human circulation at near physiological concentrations. Panel A: Nitrite infusion increases blood flow 22% in ten normal volunteers. Panel B: Increase in blood flow occurs at rest, during exercise and during exercise with NO synthase inhibition and L-NMMA infusion. Panel C: Blood flow increases with regional nitrite concentrations of 2.5 μM at rest and 900 nM during exercise. Panel D: Blood flow increases during nitrite infusions are associated with the formation of iron-nitrosyl-hemoglobin from artery-to-vein. Figure reproduced with permission from Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, and Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498-1505, 2003.

Hemoglobin as an allosterically and electronically regulated nitrite reductase

During nitrite infusions into the brachial artery we observed the artery-to-venous formation of iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO) suggesting that nitrite was being reduced to NO rapidly within one half-circulatory time (5). An analysis of the iron-nitrosyl-hemoglobin levels during all experimental conditions revealed a striking inverse correlation with oxyhemoglobin saturation, i.e. as hemoglobin deoxygenated more NO formed. These physiological observations were consistent with a reaction between nitrite and deoxyhemoglobin to form NO as described by Doyle and colleagues in 1981 (9):

$NO_2^- + HbFe^{II}$ (deoxyhemoglobin) $+H^+ \rightarrow$ NO (nitric oxide) + $HbFe^{III} + OH^-$

Much of the formed NO is then captured as iron nitrosyl-hemoglobin ($HbFe^{II}$-NO) on viscinal hemes and measured as a "dosimeter" of NO production in venous blood:

NO + $HbFe^{II}$ (deoxyhemoglobin) $\rightarrow$ $HbFe^{II}$-NO (iron-nitrosyl-hemoglobin)

Consider the potential physiological implications of this simple equation. The reaction requires deoxyhemoglobin and a proton, providing oxygen and pH sensor chemistry, and generates NO, a potent vasodilator. Methemoglobin formed during the reaction will not autocapture and inactivate the NO formed. In additional experiments we found that nitrite, red cells (or hemoglobin), and hypoxia were required for in vitro hypoxic vasodilation of rat aortic rings. Indeed, in the presence of hypoxia and erythrocytes (conditions never tested in historical aortic ring bioassay studies) nitrite now vasodilated aortic rings at physiological concentrations of 200-500 nM (Figure 4) (5).

Using an in vitro aortic ring bioassay systems designed by the Patel lab to simultaneously measure vessel force tension and oxygen tension, we find that vasodilation is measureably potentiated by as low as 200 nM nitrite under hypoxic conditions (5). Importantly, these studies reveal that nitrite-red blood cell dependent vasodilation is initiated at an oxygen tension around the intrinsic hemoglobin P_{50} (PaO_2 of 40 mm Hg for rat erythrocytes and 30 mm Hg for human erythrocytes). In ongoing unpublished work from four laboratories (the Gladwin, Patel, Kim-Shapiro, and Hogg groups) we have now found that this vasodilation occurs as hemoglobin unloads oxygen to 50% saturation, and that this vasodilation is mediated by a maximal nitrite reductase activity of hemoglobin allosterically linked to its intrinsic P_{50}. This maximal reductase activity is allosterically regulated as oxygen binding to one heme decreases the redox potential of the other hemes in the tetramer, thus increasing the ability of the hemes to donate an electron and reduce nitrite. An ideal balance of available deoxyhemes for nitrite binding and oxyhemes - required to lower redox potential of the vacant hemes - is met at the 50% hemoglobin saturation (the P_{50}). Indeed the measured rate of nitrite reduction by hemoglobin is maximal at a hemoglobin-oxygen saturation between 40-60%. Such a maximal reductase activty at P_{50} is biochemically consistent with a role in hypoxic vasodilation because physiological studies demontrate an onset of hypoxic vasodilation at 40-60% hemoglobin oxygen saturation (75).

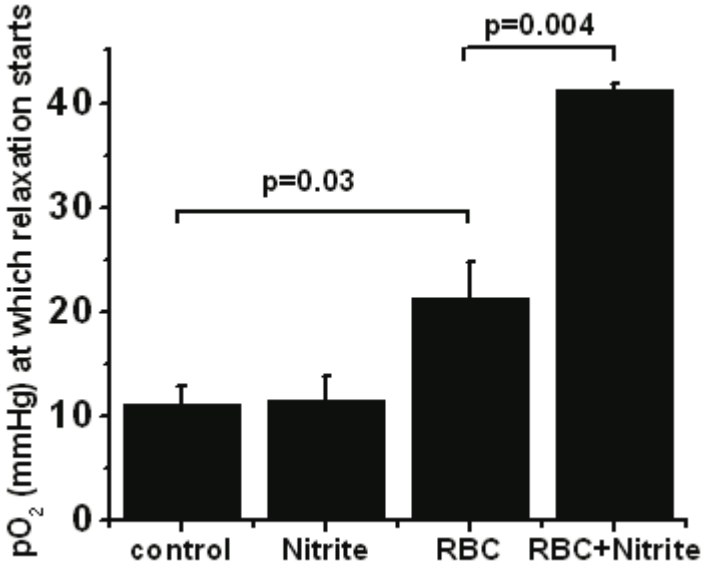

Figure 4. In the presence of hypoxia and erythrocytes nitrite vasodilates aortic rings at a PaO_2 of 30-40 mm Hg. Using an in vitro aortic ring bioassay system designed by the Patel laboratory to simultaneously measure vessel force tension and oxygen tension, we find that vasodilation is measureably potentiated by nitrite under hypoxic conditions. While control rat aortic rings and nitrite alone vasodilate at an oxygen tension of approximately 10 mm Hg, nitrite and red blood cells vasodilate at an oxygen tension around the intrinsic hemoglobin P_{50} (PaO_2 of 40 mm Hg for rat erythrocytes as shown in this figure and 30 mm Hg for human erythrocytes - data not shown). This experiment utilizes 2 μM nitrite and 0.3% red cell hematocrit. Figure based on data from Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, and Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498-1505, 2003.

INTEGRATED BIOCHEMICAL PHYSIOLOGY

Nitrite appears to fit requirements for a physiological mediator of hypoxic vasodilation as it maximally reacts with hemoglobin at 40-60% hemoglobin saturation, an oxygen tension (20-40 mm Hg) significantly higher than that required for SNO-hemoglobin deoxygenation, i.e. cysteine 93 liganded hemoglobins have very high oxygen affinities (2, 66). In the normal skeletal muscle circulation oxygen tension decreases from the A1 caliber arterioles (100 μmeter diameter) to the A4 caliber arterioles (20 μmeter diameter) to values as low as 20 mm Hg prior to the capillary circulation (87). These data suggest that much of the oxygen delivery occurs within the arterioles allowing for spacially linked oxygen delivery and vasomotor control. Additional mechanisms suggest that NO or ATP delivery to the capillary circulation produces retrograde intracellular propagation of vasodilating

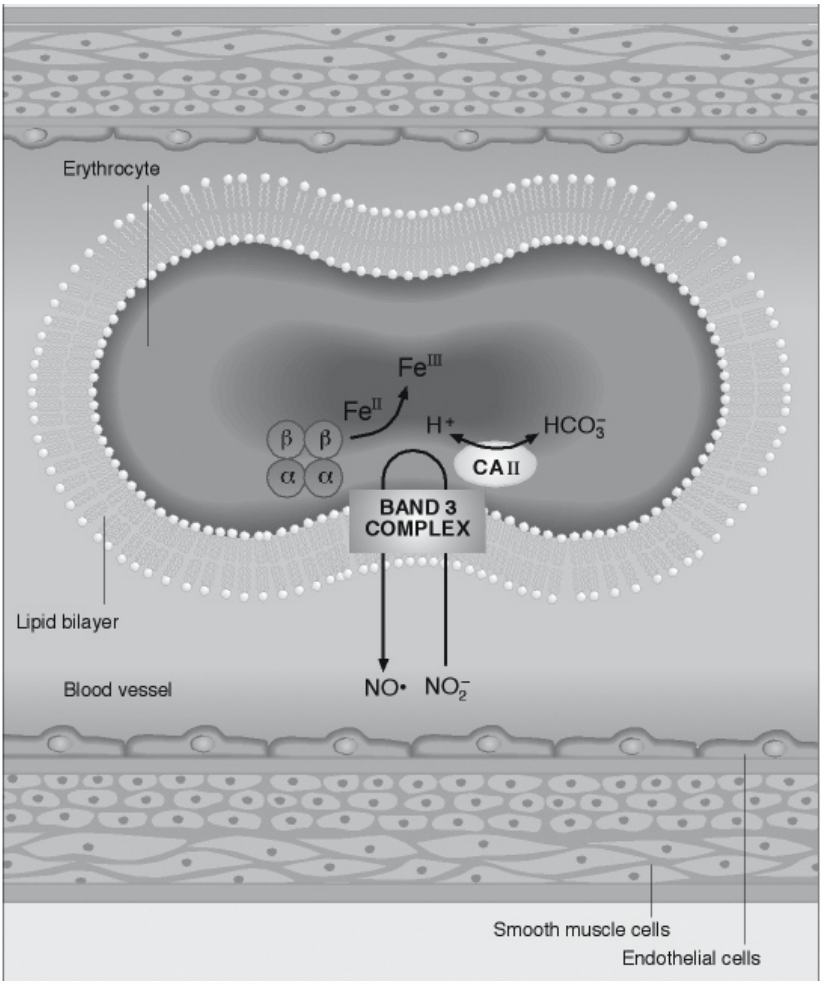

Figure 5. Putative nitrite reductase metabolon. We speculate that the erythrocyte membrane proteins provide a potential nitrite reductase metabolon function composed of deoxyhemoglobin and methemoglobin, anion exchange protein, carbonic anhydrase, aquaporin and Rh channels. Such as system would concentrate nitrite, proton, deoxyheme and highly hydrophobic channels at the membrane complex. Reproduced with permission from: Gladwin MT, Crawford JH, and Patel RP. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Radic Biol Med* 36: 707-717, 2004.

signal to the precapillary resistance vessels (80-82). Thus a maximal nitrite reductase activity at the hemoglobin P_{50} appears ideal for oxygen sensing and hypoxic vasodilation as this allosteric point is thermally, chemically and electronically responsive to physiologically relevant tissue metabolic stress. We speculate that the erythrocyte membrane proteins provide a potential nitrite reductase metabolon function composed of deoxyhemoglobin and methemoglobin, anion exchange protein, carbonic anhydrase, aquaporin and Rh channels

(16). Such as system would concentrate nitrite, proton, deoxyheme and highly hydrophobic channels at the membrane complex (Figure 5) (16). The lipophilicity and potency of NO (EC_{50} of only 1-5 nM) requires very little NO escape to regulate vasodilation, especially considering that flow is proportional to the radius to the fourth power.

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Chapter 18

EXPRESSION OF THE HETEROTRIMERIC G PROTEIN Gi AND ATP RELEASE ARE IMPAIRED IN ERYTHROCYTES OF HUMANS WITH DIABETES MELLITUS

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Abstract: Erythrocytes of humans have been reported to stimulate nitric oxide (NO) synthesis in the circulation as a consequence of their ability to release ATP in response to both mechanical deformation and exposure to reduced oxygen tension. It has been proposed that the ability of the erythrocyte to affect local vascular resistance permits it to participate in the regulation of blood flow such that oxygen delivery is matched with metabolic need. A signal transduction pathway that relates deformation and exposure to reduced oxygen tension to ATP release from human erythrocytes has been described. The heterotrimeric G protein, Gi, is a critical component of this pathway. Importantly, stimulation of Gi results in activation of adenylyl cyclase and ATP release from these cells. Recently, in a model of diabetes mellitus in rats, expression of Gi was reported to be decreased in the aorta. We report that expression of $G\alpha_{i2}$ is selectively decreased in erythrocytes of humans with type 2 diabetes (DM2) and that these erythrocytes fail to release ATP in response to incubation with mastoparan 7 (10 μM), an agent that activates Gi. These results provide support for the hypothesis that ATP release from erythrocytes of humans with DM2 is impaired and this defect in erythrocyte physiology could contribute to the vascular disease associated with this clinical condition.

Key Words: mastoparan 7, heterotrimeric G proteins, western analysis

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INTRODUCTION

Previously, we reported that human erythrocytes release ATP in response to both mechanical deformation (28,29,30) and exposure to reduced oxygen tension (3,8,9). In the vasculature, P_{2y} purinergic receptors are found on endothelial cells (5,7,16). Binding of ATP to these receptors results in the synthesis of NO (5,16). We have proposed that ATP released from the erythrocyte acts locally on endothelial cells to evoke NO release which, in turn, affects vascular caliber permitting the erythrocyte to participate in local regulation of the circulation. Indeed, the erythrocyte has been suggested to be an important determinant of the matching of oxygen supply with tissue metabolic demand (8,9,11).

The fact that ATP does not readily cross cell membranes suggests that its release from erythrocytes requires a specific mechanism. We reported that, in human erythrocytes, Gs (22) and Gi (21,23), adenylyl cyclase (30), cyclic AMP-dependent protein kinase (PKA) (30) and the cystic fibrosis transmembrane conductance regulator (CFTR) (27) are components of a signal-transduction pathway that relates physiological stimuli such as mechanical deformation or exposure to reduced oxygen tension to ATP release (Figure 1).

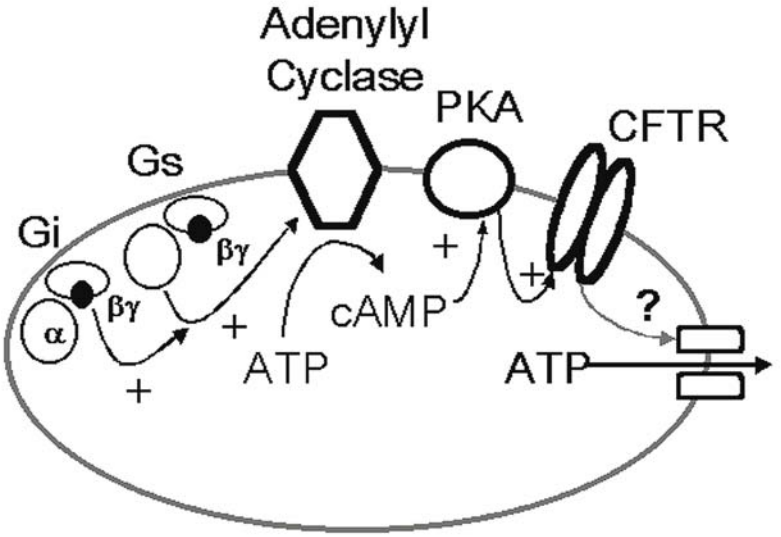

Figure 1. Proposed pathway for regulated ATP release from human erythrocytes. Abbreviations: Gs and Gi - heterotrimeric G proteins; ATP - adenosine triphosphate; cAMP - 3'5'-cyclic-adenosine monophosphate; PKA - protein kinase A; CFTR - cystic fibrosis transmembrane conductance regulator**; ?** - a yet unidentified conduit for ATP release; + - stimulates.

Recently, altered expression of one of the components of this pathway, Gi, has been reported to occur in an animal model of diabetes mellitus (12). In rats made diabetic with streptozotocin, significant reductions in the expression of the heterotrimeric G-proteins, $G\alpha i_2$ and $G\alpha i_3$ were detected in the aorta (12). This finding is of particular interest as we have demonstrated that Gi is a necessary component of a signal-transduction pathway for ATP release from erythrocytes of humans (21,23) in response to mechanical deformation

and exposure to reduced oxygen tension. Importantly, Gi activation results in stimulation of adenylyl cyclase and ATP release from these erythrocytes (21,23).

The finding that activation of Gi is a critical component of a signal transduction pathway for ATP release from erythrocytes in response to physiological stimuli, coupled with reports that expression of this G protein is reduced in experimental diabetes, suggested to us that erythrocytes of humans with diabetes might have a defect in ATP release from erythrocytes that could, ultimately, lead to a decreased stimulus for endogenous NO synthesis. Here, we investigated the hypothesis that expression of the heterotrimeric G protein, Gi, is reduced in erythrocytes of humans with type 2 diabetes (DM2) and that this defect is associated with decreased ATP release in response to an agent that activates that G protein, mastoparan 7 (MAS 7) (15,21,23).

METHODS

Generation of washed erythrocytes: Blood was obtained from healthy humans as well as humans with type 2 diabetes mellitus (DM2) by venipuncture (antecubital fossa). Blood was collected into a heparinized syringe and centrifuged at 500 x g at 4°C for 10 min. The plasma and buffy coat were discarded. Erythrocytes were resuspended and washed x 3 in buffer (in mM, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl 1.2 $MgSO_4$, 21.0 tris(hydroxymethyl)amino methane, 5.5 glucose with 0.5% bovine serum albumin (final pH 7.4)). Cells were prepared on the day of use.

ATP assay: ATP was measured by the luciferin-luciferase technique (3,21-23,27-30) in which the amount of light generated by the reaction of ATP with firefly tail extract is dependent on ATP concentration. Sensitivity was augmented by addition of synthetic D-luciferin. A 200 µl sample of erythrocyte-containing solution (or ATP standard) was injected into a cuvette containing 100 µl firefly tail extract (10 mg/ ml distilled water, FLE 50, Sigma, St. Louis, MO) and 100 µl of a solution of synthetic D-luciferin (5 mg/10 ml distilled water, Sigma, St. Louis, MO). The peak light efflux was determined using a luminometer (Turner Designs, Sunnyvale, CA, model 20/20). A standard curve was obtained on the day of each experiment. Values for ATP were normalized to an erythrocyte count of 4 x 10^5 cells/mm^3.

Measurement of hemoglobin: To exclude the possibility that concentrations of extracellular ATP represent that released by the lysis of erythrocytes, after ATP determinations, erythrocyte suspensions were centrifuged at 500 x g at 4°C for 10 min and the presence of hemoglobin in the supernatant was determined by light absorption at a wavelength of 405 nm (33). The assays for ATP and hemoglobin have similar sensitivities such that an increase in ATP, if unaccompanied by an increase in hemoglobin, represents active ATP release from erythrocytes and is not the result of cell lysis. If increases in hemoglobin were detected, the study was excluded.

Preparation of erythrocyte membranes: Erythrocyte membranes were prepared as follows. All lytic steps were conducted at 4°C. Washed RBCs (2 ml) were added to 200 ml of hypotonic buffer (in mM; 5 Tris-HCl, 2 EDTA with pH adjusted to 7.4) and stirred vigorously for 20 min. The mixture was centrifuged at 23,300 x g for 15 min. The super-

natant was discarded and the membranes were re-suspended in buffer. The mixture was centrifuged at 23,300 x g for 15 min and the supernatant discarded. The membranes were pooled, re-suspended in buffer, and centrifuged again at 23,300 x g for 15 min. The protein concentration was determined with the BCA Protein Assay (Pierce).

Identification of the α subunit of heterotrimeric G proteins in erythrocyte membranes: Erythrocyte membranes were solubilized in SDS sample buffer (0.277 M sodium dodecyl sulfate (SDS), 60% glycerol, 0.4 M dithiothreitol, 0.25 M Tris HCl, and 0.004% bromophenol blue) and heated (5 min, 100°C) before loading onto a precast 4-20% gradient Tris-HCl Ready Gel (Bio-Rad). Gels were subjected to electrophoresis at 150 V for 1.5 h with buffer containing 25 mM Tris, 192 mM glycine and 0.1% w/v SDS, pH 8.3. After electrophoresis, proteins were transferred for 1 h on ice onto a polyvinylidene difluoride (PVDF) membrane with transfer buffer (25 mM Tris and 192 mM glycine with 20% v/v methanol at pH 8.3) at 100 V. PVDF membranes were blocked (overnight, 4 °C) with 5% non-fat dry milk in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), then incubated with antibodies directed against the alpha subunit of Gs, (rabbit polyclonal), Gi_2 (mouse monoclonal), and Gi_3 (rabbit polyclonal) (Biomol). In addition, membranes were incubated with antibody directed against ß-actin, a structural protein in erythrocytes. PVDF membranes were then incubated (4 h, 25 °C) with donkey anti-rabbit or sheep anti-mouse IgG linked to horseradish peroxidase (Amersham Biosciences) as the secondary antibody in 1% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween 20. After labeling with the secondary antibody, PVDF membranes were exposed to enhanced chemiluminescence using ECL (Amersham Biosciences).

Determination of relative amounts of G protein α subunits present in erythrocyte membranes: Amounts of membrane protein loaded on the gels were standardized by determination of the protein content of each preparation prior to loading and by determination of the amount of ß-actin present in each sample on separate gels with protein diluted 5 fold. Amounts of G protein in individual membrane preparations were calculated as the ratio of G protein to β-actin . Values measured in humans with DM2 are expressed as the percentage of that detected in the membranes of healthy humans run on the same gel. All studies were performed in duplicate.

Study of Patients with Diabetes Mellitus Type 2 (DM2): Patients with diabetes mellitus type 2 (DM2) were identified by physicians in the Endocrine Clinic at Saint Louis University. A history form was completed for each individual studied (both healthy volunteers and humans with DM2). The information obtained included a medication history, a detailed listing of all medications and age, as well as the most recent HbA1c level (values within four weeks of blood removal). The mean age of healthy humans and humans with DM2 was 40±5 and 51±5 respectively. Patients with DM2 (n=9) were treated with insulin (n=8), lipid lowering agents (n=8) oral hypoglycemic agents(n=6), anti-platelet agents (n=6), diuretics (n=5), β blockers (n=5), angiotensin converting enzyme inhibitors (n=5) and calcium channel blockers (n=3). All record keeping was in strict compliance with HIPPA regulations.

Incubation of erythrocytes with MAS7: After washing, erythrocytes were diluted with wash buffer (see above) to achieve a hematocrit of 20%. Erythrocytes of healthy humans or humans with DM2 (HbA1c values of 8 or greater, range 8.1 to 11.8) were incubated with either mastoparan 7 (MAS 7, activator of Gi, 10 μM) or its vehicle (saline). ATP release was measured at 5, 10 and 15 minutes after addition of MAS 7 or buffer. ATP was measured by adding 200 μl of erythrocyte suspension diluted 250 fold to the luciferin/luciferase mixture. The amount of free hemoglobin in each sample was measured.

Statistical methods: Statistical significance between experimental periods was determined with an analysis of variance. In the event that the *F* ratio indicated that changes had occurred, a least significant difference test was used to identify individual differences. *P* values of 0.05 or less were considered statistically significant. Results are reported as means ± SE.

RESULTS

Quantification of heterotrimeric G protein α subunits in erythrocyte membranes: Erythrocyte membranes of humans with DM2 were found to contain amounts of Gsα (n=4, HbA1c=12.3±1.8) and $G\alpha i_3$ (n=5, HbA1c=12.2±1.4) that were not different from those found in heathy humans (n=6 and 5, respectively) (Figure 2). In contrast, expression of $G\alpha i_2$ (n=5, HbA1c=12.2±1.4) was reduced to 56±2 % of that found in healthy humans (n=8) (Figures 2 and 3).

ATP release from erythrocytes in response to MAS 7: Incubation of erythrocytes of healthy humans with 10 μM MAS 7 resulted in 7 ± 1 fold increase in ATP release (n=8, *P*<0.01) (figure 4). In contrast, incubation of erythrocytes of humans with DM2 (HbA1c= 10.1± 0.7, n=5) with MAS 7 did not result in increased ATP release (Figure 4).

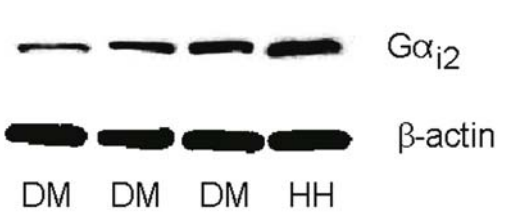

Figure 2: Measurement of the heterotrimeric G protein $G\alpha i_2$ and β-actin in erythrocyte membranes in a healthy human (HH) and humans with diabetes mellitus type 2 (DM). Membranes were prepared and the proteins were resolved using a 4-20% gradient TRIS-HCl pre-cast gel. Protein was transferred to a PVDF membrane and incubated with a mouse monoclonal anti-$G\alpha i_2$ antibody.

DISCUSSION

In humans with diabetes mellitus (DM), cardiovascular disease accounts for 66% of deaths (14,20). The most prevalent form of human DM (90-95% of individuals) is classified as type 2 (DM2) (20). DM2 is characterized by peripheral insulin resistance with an insulin secretory defect that varies in severity. It occurs typically in individuals over

40 years of age who have a family history of diabetes. Over 90% of these individuals are obese. Patients with DM2 retain the ability to secrete some endogenous insulin, therefore, they do not develop diabetic ketoacidosis. Thus, these individuals may require insulin and/or oral hypoglycemic agents to control blood sugar levels, but do not depend on them to prevent ketoacidosis.

The observation that vascular reactivity is altered in humans with DM2 is widely accepted (14,20). Indeed, both endothelium-dependent and -independent vasodilation is impaired in humans with DM2 (1,19,34,35). Impaired vasodilation in DM2 has been attributed to decreased NO synthesis (34), increased NO degradation (1) and/or to abnormalities in the vascular smooth muscle (35). Mechanism(s) notwithstanding, NO-mediated vasodilation is reduced in humans with DM2. It is possible that, in addition to the endothelium, other sources of endogenous NO may be compromised in these individuals. In addition, changes in erythrocyte physiology have also been reported to be present in humans with DM.

Erythrocytes of patients with DM have been reported to have reduced glutathione concentration (6) although this observation has not been confirmed by others (17). However, it is agreed that the oxidant stress to which erythrocytes are exposed is increased in a high glucose environment (6,17). It has been hypothesized that this oxidant stress leads to increased glycation of erythrocyte proteins (4). Glycated hemoglobin (HbA1c) has been accepted as a measure of the degree of glycemic control in patients with diabetes such that the lower the HbA1c level, the better the glycemic control (26,32). Indeed, HbA1c levels correlate with diabetic complications (17,32), i.e., better glycemic control is associated with reduced complications.

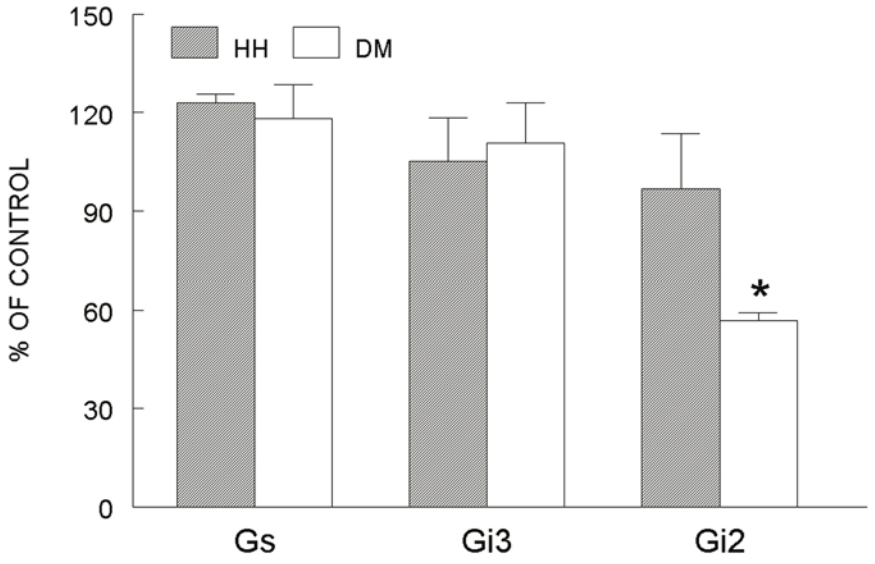

Figure 3. Expression of heterotrimeric G protein α subunits in erythrocyte membranes of healthy humans (HH, cross hatched bars) and humans with diabetes mellitus type 2 (DM, open bars). Numbers of humans studied are: Gs, 5 HH and 4 DM; Gi3, 4 HH and 5 DM and Gi2, 7 HH and 5 DM. Values are calculated as the % of the amount of G protein α subunit found in a control human erythrocyte preparation. Values are the mean ± SEM. *, $p<0.05$ compared to all other groups.

In addition to glycated hemoglobin, several properties of the membranes of RBCs of patients with diabetes differ from those of healthy humans (13,24,25). For example, exposure of erythrocytes to increased concentrations of D-glucose results in altered conductivity of the cell membrane (13). Erythrocytes of diabetics have also been reported to be less deformable that those of healthy humans (18). More important to this study, erythrocytes of patients with diabetes demonstrate a defect in ATP release in response to osmotic stress (24) and contain increased amounts of ATP (24), the latter finding possibly reflecting a failure of those mechanisms that result in ATP release. Here we investigated the hypothesis that such a defect in ATP release from erythrocytes of humans with DM2 is the result of the failure of a component or components of a signal transduction pathway for ATP release.

As stated above, we have defined a signal transduction pathway that relates physiological stimuli to ATP release from erythrocytes of rabbits and humans (figure 1). Recently, altered expression of one of the components of this pathway, Gi, has been reported in an animal model of diabetes (12). Thus, in rats made diabetic with streptozotocin, significant reductions in the expression of the heterotrimeric G-proteins, Gαi2 and Gαi3 were detected in the aorta (12). This finding is of particular interest in that we have demonstrated previously that Gi is a necessary component of a signal-transduction pathway for deformation- and reduced oxygen tension-induced ATP release from erythrocytes of healthy humans (21,23).

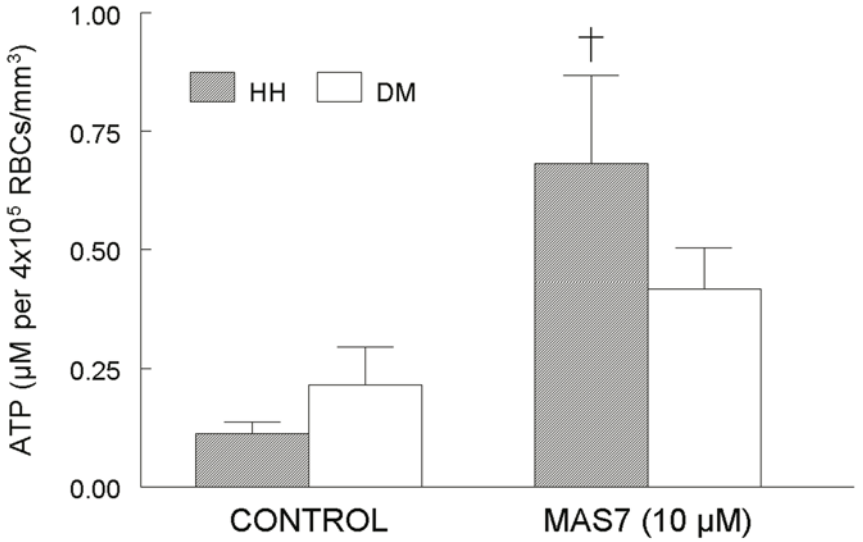

Figure 4. Effect of mastoparan 7 (MAS7) on ATP release from erythrocytes of healthy humans (HH, n=8) and humans with diabetes mellitus type 2 (DM, n=5). Washed erythrocytes were incubated with MAS7 or its vehicle (saline, control). ATP release was measured at 5, 10 and 15 min after MAS7 and the maximal response is reported. Values are the mean ± SEM. †, $p<0.05$ compared to respective control.

It is now recognized that, in addition to the α subunits of heterotrimeric G proteins, the βγ subunit is capable of activating at least three of eight membrane associated isoforms of adenylyl cyclase, specifically, subtypes II, IV and VII (table 1) (10,31). The heterotri-

meric G proteins most clearly associated with this property are of the Gi/o subclass (31). The ability of the βγ subunit to activate adenylyl cyclase subtypes II, IV and VII has been shown to reside with the β component of that dimer, of which five types have been defined (31). Of five β subunit types that have been identified, the ability to stimulate adenylyl cyclase has been associated with types 1, 2, 3 and 4, but not type 5 (2). We have reported that β subunits 1,2,3,and 4 are components of the erythrocyte membranes of humans (28). Thus, G proteins of both the Gs and Gi subclasses, as well as β subunits capable of stimulating adenylyl cyclase, are present in human erythrocytes.

The finding that activation of Gi is a critical component of a signal transduction pathway for ATP release from erythrocytes in response to physiological stimuli, coupled with the finding that expression of this G protein is reduced in the erythrocyte membranes of humans with DM2, suggests that erythrocytes of humans with DM2 could have a defect in ATP release from erythrocytes that leads to a decreased stimulus for endogenous NO synthesis. Thus, we conclude that defects in the ability of erythrocytes to release ATP in response to physiological stimuli could contribute to vascular disease in humans with DM2.

ACKNOWLEDGMENTS

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Chapter 19

RED BLOOD CELLS AND HEMOGLOBIN IN HYPOXIC PULMONARY VASOCONSTRICTION

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Abstract: Nitric oxide (NO) plays an important role in the modulation of hypoxic pulmonary vasoconstriction; in turn, red blood cells (RBCs) augment HPV by hemoglobin-mediated oxidation and inactivation of NO. In addition, scavenging of reactive oxygen species by RBCs may play a role in augmentation of HPV. NO delivery and/or production by RBCs does not appear to be important in the control of pulmonary vasomotor tone. This review will discuss regulation of HPV by RBCs with an emphasis on hemoglobin-NO interactions. In addition, the review will discuss how biologic (S-nitrosation) or pharmacologic (cross-linking) modification of hemoglobin may affect pulmonary circulatory-hemoglobin interactions.

Key Words: pulmonary blood flow, gas exchange, oxygen delivery

INTRODUCTION

Vasoconstriction of small pulmonary arteries and arterioles occurs in response to alveolar hypoxia. (43, 75, 78) In the presence of regional shunt or low ventilation-to-perfusion, this hypoxic pulmonary vasoconstriction (HPV) acts to divert blood flow away from hypoxic lung regions, thus minimizing the effects of lung pathology on the arterial PO_2.(13, 62). In the presence of global lung hypoxia, HPV results in pulmonary hypertension.

HPV kinetics are rapid, and HPV is undoubtedly an intrinsic property of pulmonary vascular smooth muscle. (55, 61, 81, 83) It is generally agreed that HPV is initiated by opening of L-type calcium channels and an increase in intracellular calcium (2, 4, 34, 68, 80, 87, 88). The location of the "hypoxia sensor" may be the mitochondria, but the role of reactive oxygen species (ROS) in transducing this signal is a subject of intense debate.(59, 82) Despite the localization of sensor and effector mechanisms for hypoxic pulmonary

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vasoconstriction to pulmonary vascular smooth muscle cells, extrinsic factors certainly modify the response. In particular, the pulmonary vascular endothelium modulates hypoxic pulmonary vasoconstriction through production of arachidonic acid derivatives, nitric oxide (NO), and endothelin, and perhaps other yet undefined mediators.(1) In addition, it is clear that the red blood cell is necessary for the full expression of hypoxic pulmonary vasoconstriction via a variety of potential mechanisms, including interactions with nitric oxide (NO), other reactive oxygen species, purines, and endothelial interactions. The remainder of this review will discuss the roles that red blood cells play in the modulation of hypoxic pulmonary vasoconstriction, with particular emphasis on the importance of NO and hemoglobin (Hb) in this interaction.

MODULATION OF HPV BY NO

Multiple levels of evidence suggest an important role for NO in the modulation of HPV, including marked augmentation of HPV in isolated lungs, intact animals, and human subjects after inhibition of NO synthesis, (3, 8, 12, 24, 41, 53, 66, 72, 74, 76), and in mice with targeted disruption of the endothelial nitric oxide synthase (eNOS) gene. (31) Administration of inhaled NO results in inhibition of hypoxic pulmonary vasoconstriction. (33, 67, 75) There appear to be species differences in the role that NO plays in regulation of pulmonary vascular resistance during normoxia and hypoxia, with NO playing an important role in mice, rats, rabbits, pigs, sheep, and humans, but less so in dogs.(18)

Given that NO is continually produced by pulmonary vascular endothelium and airway epithelium, it is remarkable that HPV is not tonically inhibited. The inhibitory effect of NO on HPV appears to be blunted by the capacity for red blood cells to take up and inactivate NO, as discussed below. In addition, an immediate fall in NO production during acute hypoxia due to reduced NOS activity may act to enhance hypoxic pulmonary vasoconstriction (29, 46, 70).

MODULATION OF HPV BY RED BLOOD CELLS AND HEMOGLOBIN: INITIAL STUDIES

Augmentation of HPV by blood was demonstrated by Duke et al in isolated, perfused cat lungs more than 50 years ago.(28) McMurtry et al later identified the role of the red blood cell in this response when they showed that isolated rat lungs perfused with colloid solution, plasma, or plasma plus platelets had rapidly decaying hypoxic pulmonary vasoconstriction when compared to lungs perfused with blood or plasma plus red blood cells. (58) Although they showed that cyclooxygenase played no role in augmentation of HPV by red blood cells, they were unable to identify a mechanism to explain their observations.

Later, potentiation of hypoxic pulmonary vasoconstriction by red blood cells was described in both rat and cat lungs, but not in lungs from swine and hamsters (39, 40). Later investigations by Hakim suggested that species differences in augmentation of HPV by red blood cells were due to differences in muscular pulmonary vessel size, and/or red blood cell deformability changes in response to hypoxia. (38, 39) Weissmann et al found that red

blood cells augmented HPV at very low concentrations (hematocrit of 1-5%), but with no further augmentation beyond that level. (84) However, the mechanism by which red blood cells augmented hypoxic pulmonary vasoconstriction remained unproven.

HEMOGLOBIN-NO INTERACTIONS

The biochemical interactions between NO and hemoglobin are discussed in detail elsewhere in this symposium. Briefly, there are four reactions that are of potential import to the regulation of vascular tone: 1) Oxidation of nitric oxide (NO) by oxyhemoglobin (oxyHb) to form methemoglobin (metHb) and nitrate irreversibly inactivates NO.(65) 2) Addition of NO to deoxyheme to form nitrosyl(heme)Hb in a reaction that is reversible under certain conditions.(14, 16, 35, 79) 3) S-nitrosation of Hb at the ß-cysteine 93 (ß-cys93) residue to form S-nitrosoHb (SNO-Hb).(45) This reaction is also reversible, particularly in the presence of low-molecular weight thiols such as GSH.(45, 85) It has also been proposed that the Hb S-nitrosation reaction is under the allosteric influence of deoxygenation, although this is controversial as will be discussed in a following section.(9, 46, 57, 77) 4) Reduction of nitrite by deoxyHb to form NO.(17, 27, 50) Of these reactions, the oxidation and addition reactions have the potential to limit the magnitude and duration of NO's vasorelaxant effects during hypoxia, whereas the S-nitrosation and reduction reactions have the potential to generate NO during hypoxia.

HEMOGLOBIN, NO, AND HPV

Given the large capacity for oxidation of NO by ferrous Hb, largely through irreversible oxidation to form metHb and nitrate, we postulated that red blood cells augment hypoxic pulmonary vasoconstriction by reducing local NO availability. Indeed, several studies have demonstrated augmentation of HPV by free Hb. (41, 42, 56) Furthermore, Heller et al showed that NOS inhibition by L-NAME did not further augment hypoxic pulmonary vasoconstriction in lungs perfused with buffer containing free Hb. (42)

Isolated, perfused rabbit lungs are particularly suited to the study of the interactions between red blood cells, NO, and HPV, in that they have a moderately strong pressor response to hypoxia, and also demonstrate a high concentration of NO in the exhaled breath, with the latter being highly and rapidly sensitive to changes in perfusate and alveolar conditions. (7, 15, 37) Accordingly, using this model we found that both HPV and exhaled NO varied with changes in perfusate hematocrit; HPV was augmented and exhaled NO depressed as hematocrit was increased within the physiologic range (10-30%) (Figure 1).(24) Nitric oxide synthase (NOS) inhibition with the L-arginine analog N^{ω}-nitro-L-arginine (L-NA) resulted in a reduction in exhaled NO in buffer-perfused lungs to the level of that in lungs perfused with a hematocrit of 30%, and markedly enhanced HPV in buffer-perfused lungs, but not in lungs perfused with red blood cells (Figure 2). Other investigators corroborated our findings, and showed that red blood cells but not leukocytes or platelets augment hypoxic pulmonary vasoconstriction and reduce intravascular NO in isolated rat lungs. (47)

In later studies, we provided more direct evidence that red cells augment HPV by inactivating NO. When isolated, perfused rabbit lungs were exposed to a very low concentration

of free Hb (~4 μM), exhaled NO was reduced and HPV was augmented to a similar degree as when perfusate contained an approximately 400 fold greater concentration of intraerythrocytic Hb (perfusate hematocrit 30%) (Figure 2). (19)

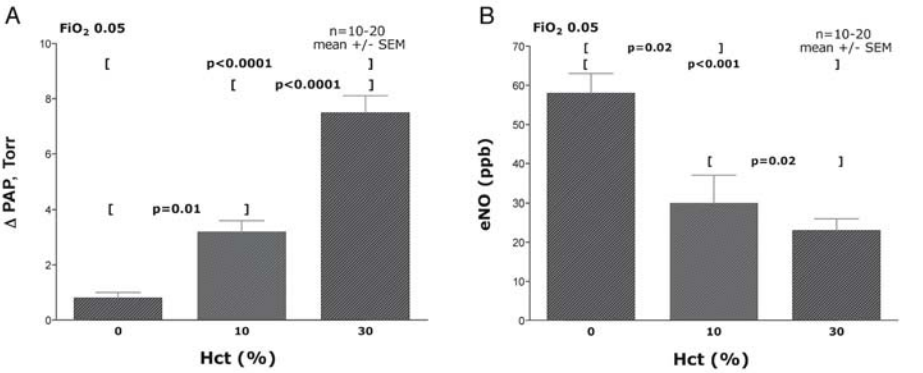

Figure 1. A. Hypoxic pulmonary vasoconstriction (HPV) represented as the change in pulmonary artery pressure (ΔPAP) from baseline 5 minutes after initiation of hypoxic ventilation. HPV increases with the addition of red blood cells to the perfusate in a "dose-dependent" fashion. B. Exhaled NO (eNO) in lungs perfused with buffer, or with buffer containing red blood cells at hematocrits of 10 and 30%.

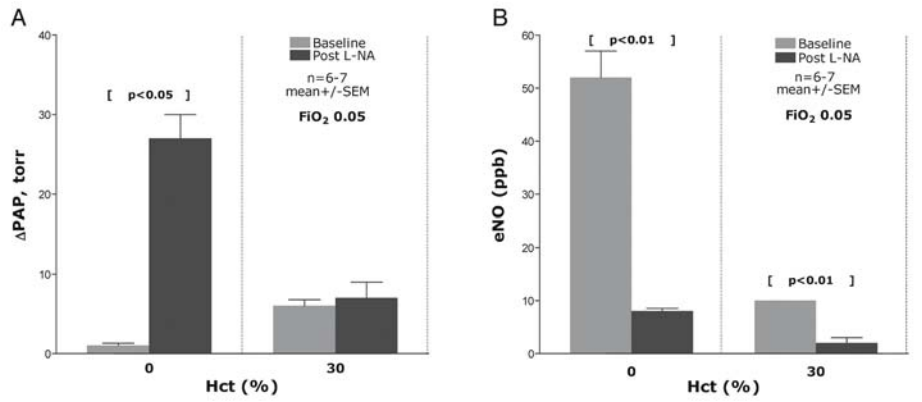

Figure 2. A. HPV before and after nitric oxide synthase (NOS) inhibition in lungs perfused with buffer, or buffer plus red blood cells (Hct 30%). NOS inhibition augments HPV in buffer but not red cell-perfused lungs. B. Exhaled NO (eNO) before and after NOS inhibition in lungs perfused with buffer, or buffer plus red blood cells (Hct 30%). NOS inhibition reduces exhaled NO in both conditions, but the change is more dramatic in buffer-perfused lungs.

Treatment of free Hb with potassium ferricyanate to form cyanometHb to prevent all interactions of heme with NO (oxidation and nitrosylation), prevented augmentation of HPV and reduction of exhaled NO in this model (Figure 3). (21) These data provide convincing evidence that the red cell augments HPV by Hb-mediated NO inactivation. The data also

illustrate the remarkable ability of the red blood cell membrane to protect NO from degradation by heme.

There may be a Hct threshold effect for inactivation of NO and augmentation of HPV by red blood cells. Loer and Peters demonstrated that HPV was augmented in isolated rabbit lungs when perfusate hematocrit was increased from 0-1% to 16-18% and then to 34-36%, but an increase in hematocrit to 49-51% did not further increase HPV. (54) Alternately, a high hematocrit may have other effects on the pulmonary circulation (see discussion below) that negate the scavenging capacity of Hb for NO.

Limited *in vivo* work supports the notion that red cells are important in the modulation of HPV. In anesthetized rabbits with lobar atelectasis, hemodilution caused a relative increase in intrapulmonary shunt and blood flow to the collapsed lung.(20) This increase in shunt could not be explained by the purely physical effects of reduced blood viscosity, and thus likely represented a reduction of hypoxic pulmonary vasoconstriction in the atelectatic lung. Anesthetized dogs ventilated with a hypoxic gas mixture exhibited an approximately 50% reduction in HPV when they were hemodiluted from a Hct of 40% to 20%.(48)

No studies have directly tested the hypothesis that red blood cells modulate HPV in humans. However, indirect evidence is demonstrated by the observation that polycythemic patients with chronic hypoxemic lung disease have attenuated vasodilation in response to acetylcholine infusion (25). Vasodilation by acetylcholine can be restored by a reduction in hematocrit. In summary, these data suggest that augmentation of hypoxic pulmonary vasoconstriction by red blood cells/Hb is a measurable and likely important physiologic effect.

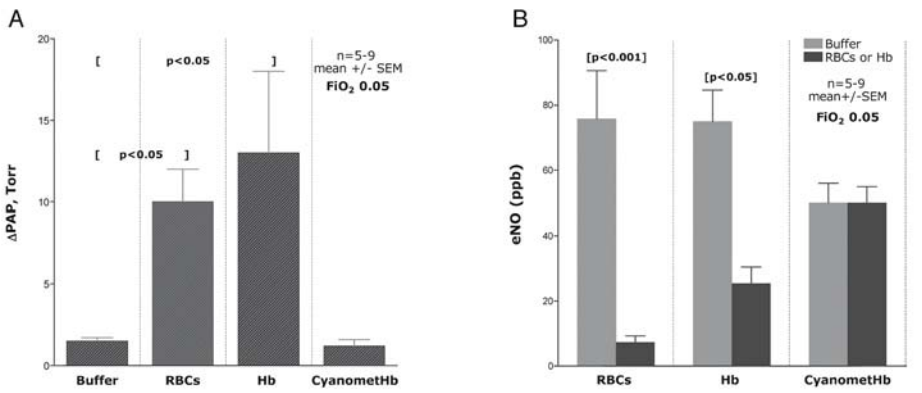

Figure 3. A. HPV in lungs perfused with buffer, or buffer plus RBCs (Hct 30%), buffer plus free Hb (3.6 μM), or buffer plus Hb treated with potassium ferricyanate (cyanometHb) to block oxidation and addition reactions of Hb with NO. Free Hb augments HPV at a concentration 400-fold less than that found in RBCs at Hct 30%, whereas cyanometHb has no effect on HPV. B. Exhaled NO in lungs before and after adding the RBCs, Hb, or cyanometHb to the perfusate. RBCs and Hb reduce eNO, whereas cyanometHb does not.

As noted above, we found that concentrations of free Hb as low as 4 μM resulted in evidence of NO inactivation and augmentation of HPV. Free Hb concentrations of this magnitude and higher have been described in patients undergoing massive blood transfusion (64) and with sickle cell disease (69). This suggests the potential for augmented pulmonary (and

systemic) vasoconstriction in these and other conditions associated with hemolysis.

As mentioned previously, the role of the red cell in modulation of HPV appears to predominantly one of augmentation, largely due to inactivation (oxidation) of NO by oxyHb. However, recent work suggests that red blood cells may have the capacity to release NO under certain conditions. The S-nitrosylhemoglobin hypothesis suggests that SNO-Hb is formed in the pulmonary circulation in conjunction with uptake of O_2 by Hb, and that Hb then releases SNO in hypoxic systemic tissues, where it either directly or through NO causes local vasodilation. This Hb-NO-O_2 linkage would act to maximize O_2 delivery to hypoxic tissues. How SNO-Hb might effect HPV is unclear; since SNO-Hb is putatively formed as Hb takes on oxygen in the lung, it would appear that little SNO-Hb would be formed in hypoxic lung units and that there would be little or no effect of SNO-Hb biochemistry in regions where HPV is operant. However, the isolated, perfused lung model provides the unique environment to test the effects of SNO-Hb on vascular smooth muscle and NO production. Hypoxic ventilation reverses the normal flux of O_2 from alveolus to blood, and thus mimics the movement of O_2 to tissues in the systemic circulation; this will result in deoxygenation of Hb and provide conditions for the release of NO from SNO-Hb. The pulmonary circulation also constricts in direct response to hypoxia, unlike the systemic circulation, and thus provided a strong signal for the measurement of vasodilator activity. Given these considerations, we utilized the isolated, perfused lung to study the allosteric and vasodilatory properties of SNO-Hb.

In isolated, perfused rabbit lungs SNO-Hb augmented HPV and reduced exhaled NO to the same magnitude as non-S-nitrosated oxyHb. (21) The rate of release of SNO from Hb was unaffected by hypoxia, and metHb levels increased as SNO-Hb levels fell. One limitation of this study was that the perfusate PO_2 was well above the p50 for SNO-Hb, and may have not provided a strong stimulus for Hb deoxygenation and SNO release; therefore, we repeated the study using an isolated, perfused rat lung model in which the perfusate PO_2 was reduced to an average of 11 Torr (22). Once again, SNO-Hb augmented hypoxic pulmonary vasoconstriction to the same magnitude as free oxyHb (Figure 4), and deoxygenation had no influence of the rate of loss of SNO from Hb. In both studies, glutathione (GSH) accelerated the rate of SNO loss from Hb and commensurate metHb formation. In aggregate, these data suggest that the oxidation reaction of NO with heme predominates over any effect of release of SNO/NO from Hb, and that there is not an allosteric influence of deoxygenation on the release of SNO from Hb.

It is likely that SNO-Hb encapsulated by the red cell membrane behaves entirely differently than when in the free form. Thus, we tested whether red blood cells loaded with supraphysiologic concentrations of SNO-Hb (SNO-RBCs) would vasodilate the pulmonary circulation when hypertension was induced by hypoxic ventilation or infusion of a thromboxane analog during normoxic ventilation. Using an isolated, perfused rat lung model, we found that “SNO-RBCs” did indeed reduce the pressor response to hypoxia, but they equally reduced the pressor response to thromboxane infusion during normoxia (Figure 5).(23) Moreover, the inhibitory concentration of SNO-Hb within red cells was several-fold higher than that measured the mammalian circulation, and the loss of SNO from red cells was not affected by the perfusate PO_2. These data provide further evidence against the allosteric regulation of SNO-Hb formation and degradation, and suggest that SNO-Hb is unlikely to be an important regulator of pulmonary vascular tone.

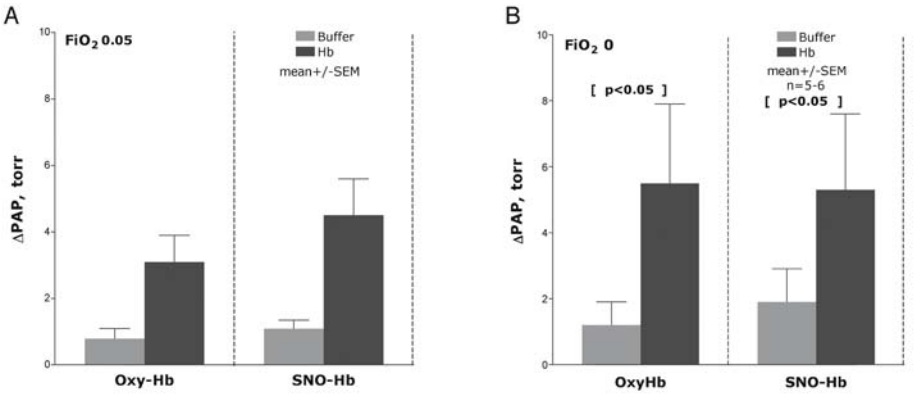

Figure 4. A. HPV before and after addition of oxyHb or SNO-Hb to buffer perfused rabbit lungs. Perfusate PO_2 in these experiments was approximately 60 Torr. OxyHb and SNO-Hb have similar effects on HPV. B. As for panel A, rat lungs. PO_2 in these experiments was approximately 11 Torr, near the p50 for free SNO-Hb.

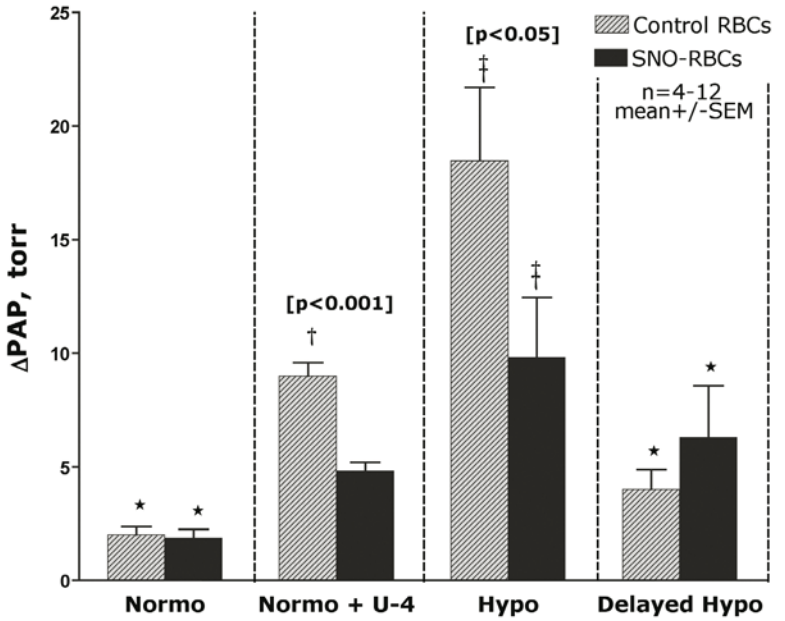

Figure 5. Effect of RBCs containing SNO-Hb (SNO-RBCs) on pulmonary artery pressure during normoxic (infusion of U-46619) or hypoxic pulmonary hypertension in isolated rat lungs, compared to the effect of control RBCs (no SNO-Hb). SNO-RBCs reduce PAP under both normoxic and hypoxic conditions, but the effect is lost at lower SNO concentrations (late hypoxia).

Nitrite has also been postulated to be an important modulator of vascular tone, serving as a storage pool for NO. NO is converted to nitrite in hypoxic conditions, and deoxyhemoglobin has been postulated to play an important role in NO generation from nitrite by serving as a nitrite "reductase".(27, 50, 63) Nitrite at low concentrations has been shown to relax systemic vascular smooth muscle in vitro, and to increase forearm blood flow in vivo.(17, 36) Furthermore, Hunter et al have shown that inhaled, nebulized nitrite reduces hypoxia-induced pulmonary artery pressure in fetal sheep.(44) However, in the latter study local lung concentrations of nitrite where likely high, and systemic blood nitrite concentrations were several times that measured in the normal circulation.

In preliminary studies, we have found that relatively low perfusate concentrations of nitrite (5 μM) reduce HPV in isolated, perfused rat lungs (Figure 6). However, addition of red cells to the perfusate attenuated the effect of nitrite on HPV, in contrast to the effect seen when nitrite is applied to aortic rings. The reasons for the discrepancy in our findings from those of others are not evident, and the role of circulating nitrite in the modulation of HPV is unclear. Nonetheless, inhaled, nebulized nitrite may have therapeutic application in the treatment of pulmonary hypertension and refractory hypoxia.

Although much of the previous discussion focuses on how the red blood cell affects NO, it is also important to recognize that NO may affect the red blood cell. NO increases red cell deformability, which in turn may have important effects on microvascular blood flow(5, 11, 51) . Given that hypoxia reduces red cell deformability (38), a combination of NO deficiency and hypoxia may have additive detrimental effects on the ability of red cells to traverse the microcirculation, and contribute to pulmonary hypertension in these conditions (26).

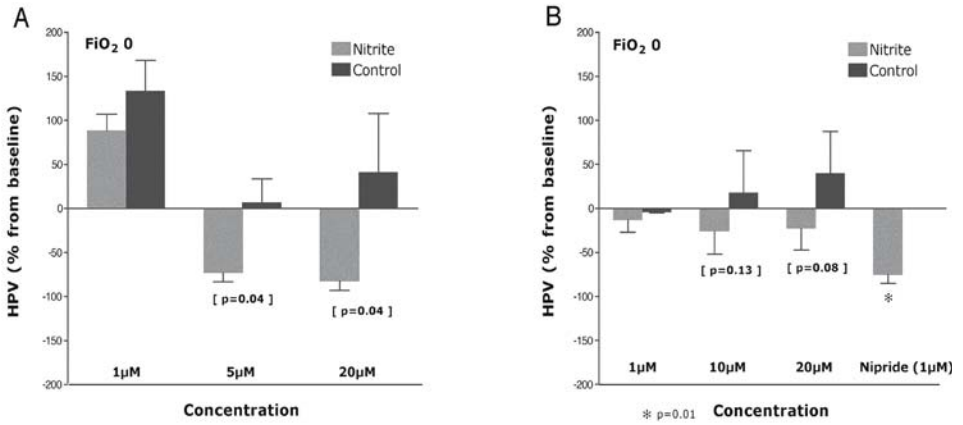

Figure 6. A. Effect of nitrite on HPV in buffer-perfused rat lungs. HPV is reported as the a percentage change from baseline response. Nitrite inhibits HPV at a concentration of 5 μM. B. Effect of nitrite on HPV in lungs perfused with buffer containing RBCs (Hct 15%). Inhibition of HPV by nitrite is lost in the presence of RBCs.

RED BLOOD CELLS AND MODULATION OF HYPOXIC PULMONARY VASOCONSTRICTION: OTHER MECHANISMS

The role of reactive oxygen species in the initiation and modulation of HPV is controversial, and the interested reader is directed elsewhere for reviews of this issue.(59, 82) Suffice it to say there is evidence that HPV may be provoked or inhibited by reactive oxygen species, and that the anti-oxidant capacity of red cells may play a role in the modulation of HPV. Red blood cells are rich in antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase. Yamaguchi et al found that lungs subjected to oxidant stress by addition of xanthine and xanthine oxidase exhibited diminished HPV, which could be restored by addition of red blood cells to the perfusate. Inhibition of SOD, but not catalase, in red blood cells prevented restoration of hypoxic vasoconstriction; addition of exogenous SOD once again restored vasoconstriction (86). Thus, inactivation of superoxide radical (but not H_20_2) by red blood cells during high oxidant stress appears to be important in maintaining hypoxic pulmonary vasoconstriction.

Kerbaul et al found that the antioxidant n-acetylcysteine restored HPV to baseline values in dogs that had undergone hemodilution to a Hct of 20%.(48) They postulated that the reduced antioxidant capacity induced by anemia was responsible for a reduction in HPV. However, no measurements of NO or oxidant stress were reported, thus limiting the implications of this study. N-acetylcysteine may reduce NO production by inhibiting arginase, which would also explain their observations.(30)

In addition to augmentation of HPV, the red cell may also have an inhibitory effect, with the former predominating under most conditions. This inhibitory effect is suggested by the observation that NOS inhibition dramatically augments HPV in buffer-perfused lungs, but not in red cell-perfused lungs (Figure 2).(24) This relative inhibition of HPV by red cells may be due to effects of viscosity. Although modeling of pulmonary arterial pressure changes with variations in blood viscosity predict a diminution of HPV as Hct falls, (48) the experimental evidence suggests the opposite. In 1975, Benumof demonstrated that increased blood viscosity inhibited HPV in intact dogs.(6) We confirmed this finding in isolated, perfused rabbit lungs, and also demonstrated that the isolated red blood cell membrane (red blood cell ghosts) inhibits HPV (Figure 7).(19) Inhibition of HPV by increased perfusate viscosity was not prevented by either cyclooxygenase or NOS inhibition, but it appears likely that increased viscosity produces shear-stress effects that have an inhibitory effect on HPV.

HEMOGLOBIN-BASED OXYGEN CARRIERS (HBOCS)

Structural modification of Hb in order to create a safe and effective HBOC is an area of intense research. One limitation of HBOCs lies in the increased capacity for any free Hb solution to inactivate NO (65). Accordingly, HBOCs cause pulmonary vasoconstriction both *in vitro* and *in vivo* (10, 52, 60, 73), an effect which appears to be due to NO scavenging (32, 49). Furthermore, genetic modification of a recombinant HBOC to reduce NO scavenging by heme prevents pulmonary vasoconstriction and augmentation of hypoxic pulmonary vasoconstriction (71).

Theoretically, inter- or intra-molecular cross-linking of free Hb may reduce the NO scavenging and vasoconstrictive effects of HBOCs by preventing Hb tetramer dissociation and extra-luminal movement of dimers. However, we found no difference in either baseline pulmonary artery pressure or hypoxic pulmonary vasoconstriction when free Hb that was intramolecularly cross-linked at the ß-cysteine 93 residue was compared to native Hb in isolated rat lungs (22). It is likely that more specific modifications of Hb to reduce or prevent NO scavenging, as described above, will be necessary to significantly limit the vasoconstrictive effects of HBOCs (71).

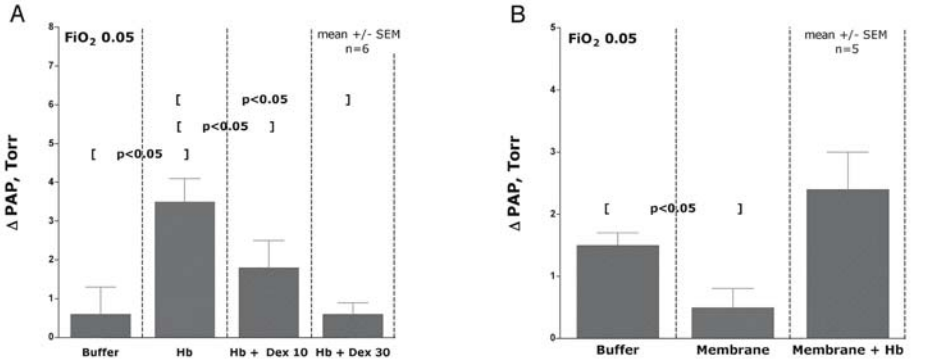

Figure 7. A. HPV in lungs perfused with buffer, or buffer containing Hb, or Hb plus dextran to raise perfusate viscosity to that comparable to a Hct of 10 and 30%. Increasing perfusate viscosity inhibits HPV. B. HPV in lungs perfused with buffer, or buffer plus RBC membrane, or membrane plus Hb. RBC membrane inhibits HPV.

CONCLUSIONS

Continuous production of NO in the lung acts to attenuate, or "brake", HPV. Red blood cells counteract this effect, predominantly by hemoglobin-mediated oxidation and inactivation of NO, and also possibly through scavenging of reactive oxygen species. Despite evidence that RBC transport and production of NO may play a role in the control of systemic vasomotor tone via hypoxic vasodilation, production of NO by RBCs does not appear to be important in the modulation of HPV. RBC-NO-HPV interactions are likely important factors in the optimization of gas exchange in health and disease, and are limiting factors in the therapeutic use of HBOCs. Further research is necessary to complete our understanding of lung-red cell interactions and their important interplay in health and disease.

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Chapter 20

DOSE-RESPONSE OF ALTITUDE TRAINING: HOW MUCH ALTITUDE IS ENOUGH?

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Abstract: Altitude training continues to be a key adjunctive aid for the training of competitive athletes throughout the world. Over the past decade, evidence has accumulated from many groups of investigators that the "living high -- training low" approach to altitude training provides the most robust and reliable performance enhancements. The success of this strategy depends on two key features: 1) living high enough, for enough hours per day, for a long enough period of time, to initiate and sustain an erythropoietic effect of high altitude; and 2) training low enough to allow maximal quality of high intensity workouts, requiring high rates of sustained oxidative flux. Because of the relatively limited access to environments where such a strategy can be practically applied, numerous devices have been developed to "bring the mountain to the athlete," which has raised the key issue of the appropriate "dose" of altitude required to stimulate an acclimatization response and performance enhancement. These include devices using molecular sieve technology to provide a normobaric hypoxic living or sleeping environment, approaches using very high altitudes (5,500m) for shorter periods of time during the day, and "intermittent hypoxic training" involving breathing very hypoxic gas mixtures for alternating 5 minutes periods over the course of 60-90 minutes. Unfortunately, objective testing of the strategies employing short term (less than 4 hours) normobaric or hypobaric hypoxia has failed to demonstrate an advantage of these techniques. Moreover individual variability of the response to even the best of living high -- training low strategies has been great, and the mechanisms behind this variability remain obscure. Future research efforts will need to focus on defining the optimal dosing strategy for these devices, and determining the underlying mechanisms of the individual variability so as to enable the individualized "prescription" of altitude exposure to optimize the performance of each athlete.

Key Words: hypoxic training, athletics, hemoglobin, erythropoietin

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INTRODUCTION

Altitude training is frequently included as a cornerstone of the training plans for elite endurance athletes(65). Since the publication of the "living high-training low" model in 1997(34), this approach to altitude training has become increasingly adopted by many teams in the United States and around the world. For example, the US speed skating team used it to great advantage in the 2002 Salt Lake City Olympic Games, living in Park City, Utah at an altitude of 2,000-2,500m and training at the Olympic Oval at 1,400m, sometimes even using supplemental oxygen to allow increased training loads(38). This approach, incorporated into a highly focused training plan, contributed to the largest number of medals awarded to the most number of skaters in US Skating history. More recently, both US medalists in the Olympic marathon at the Athens Games in 2004 incorporated LHTL into their training plans by living at Mammoth, California (2,500m), and performing intermittent high intensity interval training at low altitude in Bishop (1,250m). This effort resulted in the first ever medals awarded to US men and women athletes in the marathon in the same Olympics.

The essential features of the LHTL model appear to be living high enough, long enough to acquire the essential features of altitude acclimatization that may improve sea level performance, combined with performing enough high intensity training sessions at a low enough altitude to maintain work out quality by ensuring high rates of oxidative flux(33, 34). The key question for this presentation is how much altitude is required to stimulate a robust and sustainable adaptive response? In order to frame this discussion, it is useful to consider both exercise training and the interactive effect of altitude as a "dose" of a medication that leads to a specific physiological response. Such a "dose-response" curve is shown in figure 1.

Conceptually, at very low levels of exercise training, there is very limited adaptive response (i.e., sitting quietly, or slow walking won't lead to much of a training effect?); after some threshold level of training is achieved, with increasing doses of exercise (frequency, duration, intensity) there is a corresponding increase in the adaptive response, which can be quantified physiologically (increased maximal aerobic power, improved endurance, augmented anaerobic capacity) or by enhanced performance. In pharmacology terms, the "ED 50" would then be the "effective dose" at which 50% of the physiological effect is achieved. At some point, more training does not necessarily lead to a greater response and a plateau is achieved. Along with this dose-response relationship, there is also a toxicity curve that can be drawn. Thus for exercise, the toxic effects are relatively low at most doses of training. However there is a point at which increasing training (particularly increasing the intensity of "recovery" sessions) may lead to side effects such as staleness, over reaching or over training. Again in an analogy with a pharmacological therapy, the "LD 50" would then be the dose at which 50% of the toxic (or "lethal" -- used figuratively here...) effect is suffered. Ultimately the "therapeutic range" is the range of exercise training which is at the ED 50 or above, but also is below the rapid rise in toxic side effects.

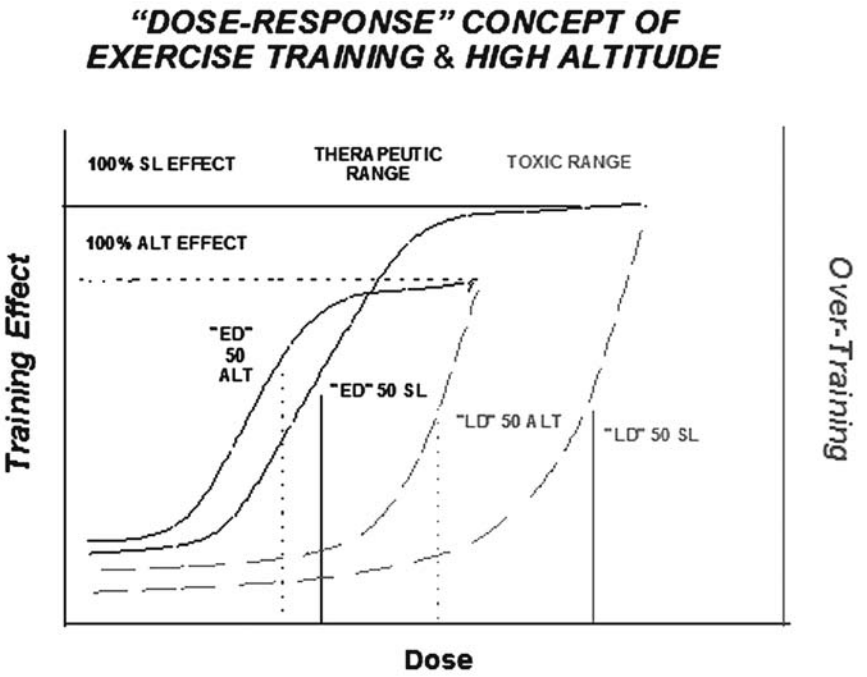

Figure 1. ″Dose-Response″ relationship between exercise training and the adaptive response at sea level (in black) and at altitude (in grey). See text for details.

Altitude has specific effects on this relationship. First of all, because exercise under hypoxic conditions is associated with prominent reductions in maximal power output, oxygen flux through mitochondrial systems, and work capacity, the peak response to endurance training under high altitude conditions is depressed(9, 20, 34, 35, 59). Because at submaximal levels, the same absolute power output at altitude is a greater fraction of the maximal capacity compared with sea level, the dose-response curve may also be shifted to the left. However the toxic effects of exercise are also more prominent and athletes may be more prone to over training while at altitude. The therapeutic range is therefore smaller with exercise training at altitude. Like exercise training, the effect of altitude by itself has its own "dose-response" curve (not shown), though each of the manifestations of the altitude acclimatization response likely has its own specific curve with different threshold, therapeutic range, and toxic profiles.

Although many possible mechanisms have been proposed as mediating a beneficial effect on performance from altitude training (including LHTL)(24), our own studies have drawn us inexorably towards the hematopoietic system(33). In addition, it is abundantly clear that increasing blood volume from direct transfusion or exogenous erythropoietin increases performance at sea level in virtually all athletes(11, 14, 15). To the best of our knowledge, this is the only effect of altitude acclimatization that has directly, and unequivocally been shown to improve sea level performance in athletes. In other words, there is no other effect of altitude acclimatization that can be (or has been) manipulated *independently* and demonstrated to improve athletic performance over a sustained period of time

(weeks).

The erythropoietic effect of altitude acclimatization is clear and compelling, particularly when the exposure is high enough, and sustained for a long enough period of time. This evidence has been reviewed recently(33) and will not be repeated here. For the purposes of the discussion regarding "dose" of altitude however, one study does bear brief discussion. For example, half a century ago, Reynafarje exposed 10 sea level natives to full time residence (i.e., 24 hr/d) at an altitude of 4,500m, and measured both plasma and red cell compartment volumes over a year at this altitude(39). After the first month, there was a small, but measurable increase in the red cell compartment (slightly less than 10%), along with a small decrease in the plasma compartment, such that total blood volume did not change. Over the subsequent half a year, the red cell mass and blood volume steadily increased, reaching its peak after 8 months of full time altitude residence (about 35% increase in red cell volume), and then increasing no further out to a year.

There are a number of key important points that deserve emphasis from this study: a) the high altitude, and long duration of continuous exposure likely make this study an example of the most vigorous erythropoietic effect that could be expected from sea level natives ascending to altitude; b) even at this "dose", the observed effect was at the limits of detection after the first 4 weeks, making it less likely that shorter duration exposures would be associated with a large effect; c) the red cell mass continued to increase for a full 8 months, despite the fact that erythropoietin levels were almost certainly near "normal" after the first few weeks. This observation emphasizes that erythropoiesis may be accelerated even in the face of apparently normal EPO levels, which are therefore elevated for the level of the red cell mass; d) plasma volume re-expanded rapidly on return to sea level, and both total blood volume and red cell volume remained above baseline for at least 2-4 weeks after descent. However after 2 months at sea level, all compartment volumes had returned to baseline.

THE LIVE HIGH -- TRAIN LOW MODEL OF ALTITUDE TRAINING

If this kind of exposure represents the maximal possible effect of altitude acclimatization, what happens with "submaximal" exposures? For the "classic' LHTL studies(13, 34, 35, 55), every effort was made for the athletes to spend as much time as possible at moderate altitude. There are a number of important design features of these studies that deserve emphasis: 1) all studies began with a 2 week "lead-in" phase where athletes were brought from their home cities to Dallas, TX (150m above sea level) for familiarization with laboratory equipment and testing procedures, and a focused period of controlled training; 2) this lead-in phase was followed by 4 weeks of training at sea level where all athletes trained together prior to randomization to bring all athletes up to an equivalent degree of training readiness. The key point to be made here is that virtually all athletes improved performance with experience and training. Therefore studies involving measures of performance which only include pre-post measurements may falsely conclude that an intervention is successful, simply because of the "training camp effect"; 3) athletes were then randomized into one of three training groups where they were exposed for 4 weeks to: a) the primary experimental group where the athletes lived at 2,500m and traveled down to a lower altitude

of 1,250m once or twice per day to train. These athletes thus spent 20-22 hr/d at moderate altitude; b) an altitude control, where the athletes lived at 2,500m together with the LHTL athletes, but did all their training at the same altitude or higher (2,500-3,000m). These athletes spent 24 hr/d at altitude; c) a sea level control where the athletes traveled to a new, training camp environment with mountainous terrain, but at sea level altitude. The volume and relative intensity of training was closely matched among groups, and followed the same pattern as the previous 4 weeks of training at sea level. All subjects then returned to sea level for post-intervention testing acutely, and then 3 weeks later.

The details of these studies have recently been summarized in some detail and will only be reviewed briefly here. (33) The essential results of these studies were as follows: 1) the athletes living for more than 20hr/d at moderate altitude (8,000 ft.) had a significant increase in erythropoietin concentration which led to a significant increase in the erythrocyte volume (blood volume -- plasma volume); neither changed significantly in a sea level control group; 2) Coincident with the increase in erythrocyte volume, there was an increase in maximal oxygen uptake in both groups living at 8,000 ft., that was proportional to the increase in erythrocyte volume, and that was not observed in the control group performing similar training in an outstanding training camp environment, but at sea level; 3) Despite an increase in $\dot{V}O_{2max}$ in both groups of subjects living at moderate altitude, only the group performing all their training at low altitude improved 5,000m racing time by 1.3%. For the LHTL athletes, this quality of training maintained muscle fiber size, myoglobin concentration, and muscle buffer capacity, all of which decreased in the athletes attempting to do all their training at moderate altitude.(57) Functionally, this preservation of muscle structure allowed an increase in both the $\dot{V}O_2$ at the ventilatory threshold, and the velocity at $\dot{V}O_{2max}$ which were present only in the high-low group.(34); 4) There was no relationship between the skill of the athlete, and the magnitude of improvement observed after 4 weeks of LHTL(55). Even elite US runners, including US Olympians achieved on average, the same 1-2% improvement in performance associated with clear evidence of accelerated erythropoiesis and increased maximal aerobic power(55).

Despite the clear superiority of living high-training low over traditional altitude or sea level training, there remains substantial individual variability in the magnitude of improvement achieved with such a regimen.(13) To address the mechanisms of this variability, we performed a retrospective review of all 39 athletes in our previous studies (34, 58) who lived at 2,500m and trained between 1,250m and 3,000m, and divided them into two groups: those athletes who improved their 5,000m race by more than the group mean ("responders"); and those that got worse ("non-responders") (13). There were no differences between these groups with respect to baseline demographic variables (age, $\dot{V}O_{2max}$, running performance, hemoglobin concentration) or many physiological variables that might determine the magnitude of the acclimatization response to altitude including pulmonary diffusing capacity, oxygen saturation either at rest, during sleep, or during exercise at 2,500m.

The key distinguishing feature between "responders" and "non-responders" was that the responders had a significantly greater increase in erythropoietin concentration which remained elevated after 2 weeks at altitude. Moreover, the responders had an increase in erythrocyte volume and increased aerobic power while the non-responders did not. This model was confirmed prospectively in a group of elite US runners(13, 55). Recently, our group has sought to determine the mechanisms of this variability in erythropoietic response to altitude (44). For example, the increase in EPO in response to acute hypobaric hypoxia

equivalent to altitude of 2,800m varies dramatically among individuals, ranging from a 40% decrease to a 400% increase in EPO after 24 hours. Although preliminary work suggested that there might be specific genetic markers on the erythropoietin gene that regulate this response (66), more detailed evaluation has been unable to identify a unique single nucleotide polymorphism (SNP) in candidate genes of the hypoxia response pathway that can explain this variability(29).

In summary, these studies demonstrated quite convincingly that: 1) the "living high-training low" model of intermittent hypoxic training works to improve sea level performance; 2) the mechanism is highly likely to be a stimulation of erythropoiesis leading to an increase in hemoglobin concentration, red cell mass, and aerobic power; 3) the effect of this increase in oxygen transport capacity is maximized by maintaining normal, sea level oxygen flux during intense exercise avoiding the down regulation of skeletal muscle structure and function that may occur in athletes who attempt to perform all their training under hypoxic conditions.

It is important to emphasize that although our results have led us to focus on the erythropoietic pathway, this was not an exclusive hypothesis at the beginning of our experiments. For example, we made detailed measurements of anaerobic capacity using the accumulated oxygen deficit method of Medbo et al(37), which in 10 years of study and more than 100 athletes, never changed over the course of any intervention(33-35). Moreover, muscle biopsy measures of buffer capacity in these same athletes failed to demonstrate an increase in buffer capacity, or increase in oxidative enzymes(34). Although we did not specifically measure the sodium-potassium pump function, which has been suggested recently to possibly contribute to altitude acclimatization(22), carefully controlled studies without harsh field conditions have failed to confirm a prominent effect of this mechanism in healthy controls (Lundby et al, personal communication) or athletes(7). Similarly, careful measures of running economy using very precise techniques have failed to identify any changes in economy in large numbers of runners(34, 35) or other athletes or healthy individuals(36). Thus the weight of evidence argues strongly for the primary effect of altitude acclimatization on sea level performance in competitive athletes being on the erythropoietic pathways.

WHAT IS THE OPTIMAL ALTITUDE?

All the published studies by our group have used an altitude of 2,500m as the primary living altitude. However one key question is what is the best living altitude for competitive athletes? To address this question, we studied 48 competitive runners at sea level, and then again after 4 weeks of living at one of 4 different altitudes: 1780m, 2,085m, 2,454m, and 2805m (n=12 each). The response to acute hypobaric hypoxia in an altitude chamber for these subjects has been published(44), though the outcome data have only been presented in abstract form(67). The key results can be summarized as follows: a) there was a progressive increase in both the mean and dispersion around the mean of the rise in EPO with increasing altitude, with the two lowest altitudes (1800m and 2100m) having significantly smaller responses than the two highest altitudes(45); b) this pattern was generally matched by the response in the field, with the major difference being that the higher altitudes resulted in a more sustained increase in EPO than the lower altitudes; c) after 4 weeks, there was

a “dose-dependent” increase in VO2 max of 8 ml, 206 ml, 308 ml, and 301 ml at 1780m, 2085m, 2454m and 2805m respectively(67); d) surprisingly, the change in performance followed a somewhat different pattern -- there was only a minimal improvement in performance (3K time) at the lowest (1.1%) and highest (1.4%) altitudes, but a much more robust improvement at the two middle altitudes (2.8% and 2.7%)(67); e) the highest altitude was associated with a significant increase in ventilation during submaximal exercise. These data suggested that there may be a relatively narrow window of “optimal dose” of altitude for competitive athletes using the LHTL model, and spending at least 20 hr/d at altitude. This altitude appears to be between 2,000 and 2,500m -- altitudes that are too low are associated with inadequate erythropoietic response, while altitudes that are too high may be complicated by other aspects of acclimatization that may be detrimental to competitive athletes, such as excessive ventilatory acclimatization, or possibly even muscle atrophy (not measured in these studies).

HOW LONG TO SPEND AT ALTITUDE?

All the studies by our group have used a minimum of 4 weeks at altitude. However many athletes use shorter training camps of 2-3 weeks for altitude training. Although we have not addressed this question specifically in our published studies, some insight can be gained from the literature. First of all, even when EPO is injected directly, there is no change in haemoglobin or hematocrit for the first week to 10 days, and then only a barely detectable increase after 2 weeks(6, 8). Thereafter, erythropoiesis accelerates, particularly between weeks 3-4 during which time hemoglobin and hematocrit increase prominently(8). Therefore altitude camps that last < 2 weeks are not likely to generate much of an improvement in aerobic power due to accelerated erythropoiesis. A compilation of published and unpublished studies carried out by the authors is shown in figure 2.

Note that there is no appreciable effect from exposures of < 2 weeks, whether to real or simulated altitude. With increasing duration of exposure, and particularly with extension of the duration at altitude to 4 weeks, there is a substantial increase in the magnitude of the erythropoietic effect. Ultimately, 4 weeks of altitude exposure above 2,000-2,500m appears equivalent in magnitude to a similar duration of low dose erythropoietin injection (50 IU/kg x 3 wk followed by 20 IU/kg x 3 wk). A recent meta analysis of studies involving varying degrees of altitude exposure by Rusko and colleagues (49)has confirmed that altitude exposures of at least 3-4 weeks are necessary to see an increase in red cell mass in the majority of athletes. The fact that the effect of 4 weeks of exposure to moderate altitude is equivalent in magnitude (erythrocyte volume, $\dot{V}O_{2max}$, and performance) to low dose erythropoietin injection provides further evidence in support of the erythropoietic pathway being a major component of the altitude effect for competitive athletes.

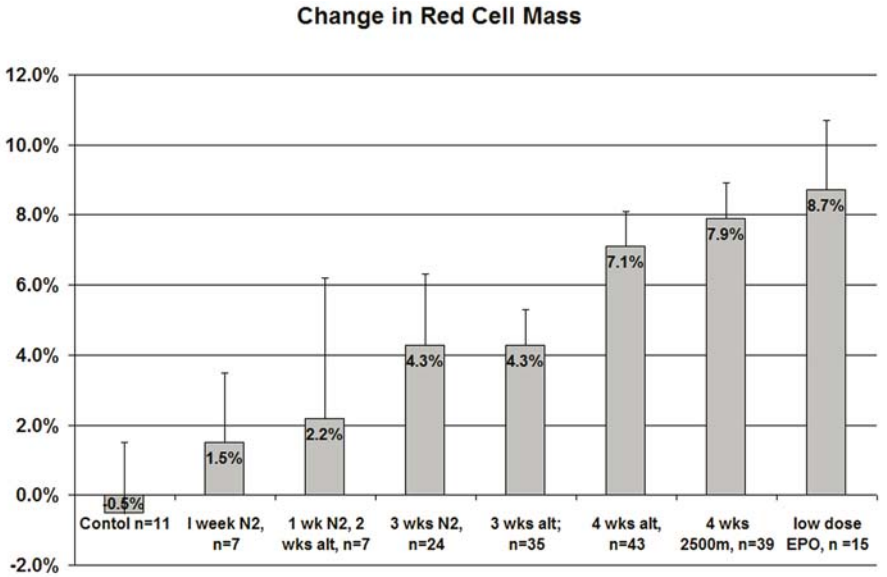

Figure 2. The columns indicate percent change in the volume of red cell mass from before to after the particular perturbation. The error bars represent the standard deviation of the percent change. Volume of red cell mass was estimated by Evans blue dye technique. A p value is associated with the column if < 0.10. All subjects were male and female currently endurance training athletes (age 18-30). Starting on the far left, Control (n=11) represents 11 athletes who were measured twice with 4-6 weeks time in between. The second column represents before and after 1 week of 12 hours per day in a nitrogen room (n=7). The third column represents the change associated with 1 week in a nitrogen room and a 2 week stay at 2100m altitude (n=7). The fourth column (n=24) represents before and after a 3 week stay (~12 hours/day) in a nitrogen room. The fifth column represents the change after a 3 week stay at natural altitude (n=35). Living altitudes ranged from 2000 to 2500m. The sixth column represents the change after a 4 week stay at natural altitude (n=43). Living altitudes ranged from 1800-2800m. The seventh column represents the change after a 4 week stay at 2500m (n=26). The far right column represents the change after a 4 week course of low dose rhEPO (50 IU/kg 3x/wk for 3 weeks, followed by 20 IU/kg 3x/wk for 3 weeks, staying below the regulatory limits for hematocrit (50) or hemoglobin (18.5) set by international organizations for the purposes of doping control).

HOW MANY HOURS PER DAY?

The compelling results from the LHTL model have led to the development of strategies designed to "bring the mountain to the athlete", particularly for athletes who do not have access to environments of sufficiently diverse altitudes to engage in a true LHTL training camp(32). A variety of approaches have been developed which involve breathing normobaric or hypobaric hypoxic gas mixtures for varying periods of time. To date, no systematic effort to examine the minimal duration of altitude exposure that is necessary to lead to sufficient sustained acclimatization responses to improve sea level performance has been accomplished.

One recent strategy that has generated a lot of interest among the athletic community has been the use of small portable hypoxia generating devices that allow individuals to breathe a hypoxic gas mixture without having to otherwise alter their living environment or behaviour patterns. Originally developed in the former Soviet Union(54) these devices are designed to provide brief periods (5 minutes) of intense hypoxia (FIO2=0.10) alternating with periods of normoxia and repeating for 70-90 minutes/d. The authors recently investigated these devices in collaboration with Randy Wilber from the USOC, Chris Gore from the Australian Institute for Sport, and Jack Daniels from Stanford University, which has been published.(31) In a randomized, double blind, placebo controlled trial, this type of intermittent hypoxia had no effect on indices of accelerated erythropoiesis, $\dot{V}O_{2max}$ or performance in highly trained runners (n=14). This study provides convincing evidence that very short periods of hypoxia, interspersed with periods of normoxia do not produce a sufficiently robust acclimatization response to increase red cell production, improve oxygen transport, or improve exercise performance (at sea level).

Most recently, the authors assembled an international group of collaborators including Ferran Rodriquez from Spain, Martin Truijens from Amsterdam, and Chris Gore from Australia to examine critically the protocol popularized by Rodriguez and colleagues(12, 46) involving a longer duration of exposure -- 3 hr/day of hypobaric hypoxia equivalent to 5,500m for 4 weeks, using 23 highly trained runners and swimmers, in a randomized, double blind, placebo controlled design (21, 47, 56, 60, 62). In order to maintain the blinding, even the subjects exposed to normobaric hypoxia experienced rapid changes in chamber pressure so that they would feel the pressure changes in their ears and other air containing spaces. With this exposure, we were able to confirm an acute increase in erythropoietin concentration, measured 2 hours after hypobaric hypoxia in the 2nd and 4th week of the experiment.(56)

However despite this transient increase in erythropoietin, there was no increase in reticulocytes, reticulocyte hemoglobin, hemoglobin concentration, soluble transferrin receptor, or total hemoglobin mass/red cell volume, measured either with carbon monoxide rebreathing or Evans blue dye(21, 56). Not only was there no evidence of erythropoietic acclimatization with this protocol (despite uncontrolled studies using relatively non-specific methods suggesting otherwise), there also was no evidence for ventilatory acclimatization, with no change in ventilation at rest or during exercise, no change in end-tidal CO_2 concentrations, and no change in the hypercapnic or hypoxic ventilatory response (HVR) which was measured weekly in runners and pre-post in swimmers(60).

This result was surprising, because the literature is more consistent with the ability of relatively short periods of hypoxia to induce ventilatory acclimatization. For example, Poulin et al have shown significant increases in the hypoxic ventilatory response (HVR), also called “short term facilitation” after only 5 days of normobaric hypoxia (1). Townsend et al, (61) using 8-10 hr/night of normobaric hypoxia have also shown an increase in HVR and a decrease in end-tidal CO_2 concentrations measured at rest. Ricart et al (40), have observed an increase in ventilation and decrease in $ETCO_2$ after only 2 hr/d of simulated hypobaric hypoxia up to 5,000m, though this increase was only manifest during submaximal exercise. It is important to note however, that all these measurements were made within the first 24 hours after the last hypoxia exposure. In contrast, our post exposure measurements were made 72 hours after completion of the last hypobaric hypoxic exposure and were unable to demonstrate lasting effects on resting or exercise ventilation. It thus may be

that the ventilatory response to such short periods of hypoxia is not very robust, and may extinguish within a relatively short period of time -- certainly not a very practical acclimatization response in terms improving sea level performance of athletes.

Lastly, in keeping with the lack of any measurable evidence of altitude acclimatization with this regimen of 3 hours/day of severe hypobaric hypoxia, we observed no improvement in sea level endurance performance in this group of athletes, either in terms of running or swimming economy(62), $\dot{V}O_{2max}$, or race times(47).

NITROGEN HOUSES OR TENTS -- "SLEEP HIGH" -- TRAIN LOW

A number of authors have attempted to administer normobaric hypoxia during sleep, both to minimize the confinement necessary with simulated altitude devices, and to allow more extended durations of exposure. An example of three such studies is shown in figure 3 suggesting a "dose-limiting" effect.

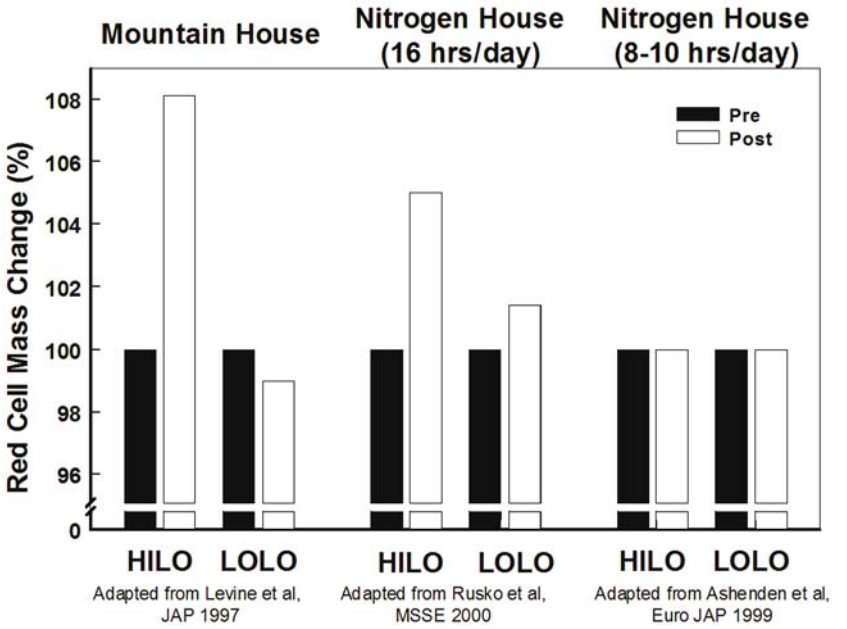

Figure 3. (HiLo=living at altitude, training at sea level; lolo=sea level control; Mountain house = field altitude 20-22hr/d). Data from (5, 32, 34).

For example, studies by the authors using 22 hours/day of altitude (2,500m) in the field ("Mountain House") for 4 weeks clearly demonstrate an erythropoietic effect of altitude exposure and an improvement in sea level performance in competitive distance runners (34). These observations have been confirmed recently by Swiss investigators in national

level orienteers, (64), in Swiss national biathletes(25), in German national junior swimmers(18), and cross-sectionally in Bolivian professional cyclists(50). Thus despite rare reports to the contrary(19) more recent publication and presentation of data from multiple different research groups around the world using the most sensitive and precise tools for assessing the hemoglobin mass (i.e., CO rebreathing) have confirmed that moderate altitude exposure for nearly 24 hr/day increases the red cell mass even in elite, highly trained athletes.

But do lesser durations of exposure also increase the red cell mass? As noted above, 3 hr/d is not sufficient to do so despite transient increases in erythropoietin(21, 56). However Finnish investigators have employed 12-16 hours/day for 4 weeks of simulated altitude in a nitrogen house (2,500m equivalent altitude), which closely replicates the results observed in the field studies (32, 48) (Fig 3). These results have been confirmed recently by Richalet and colleagues who demonstrated a similar increase in hemoglobin mass in elite French skiers and runners spending 14 hr/night in a N2 house equivalent to 3,000m for 3 weeks(10). In contrast, Australian investigators using only 8-10 hours/day of simulated altitude exposure (2,500-3,000m) for 10 days -- 21 days failed to demonstrate an increase in the red blood cell mass, though other changes in ventilation, anaerobic performance and peak power output were identified (5) (Fig 3).

Recent advances in the understanding of the biological pathways involved in the adaptive response to hypoxia have the potential to contribute substantially to this debate. For example, the principal transcriptional activator of gene expression in hypoxic cells is hypoxia-inducible factor 1 alpha (HIF-1) (51-53, 63). Under normal, well oxygenated conditions, HIF-1 is hydroxylated via a highly conserved prolyl hydroxylase (the putative cellular “oxygen sensor” in peripheral tissues), which then binds to the Von Hipple-Lindau factor, targeting the entire complex for rapid degradation via the ubiquitin- proteosome pathway (16, 27, 28). In fact, this process is so rapid, that in the presence of oxygen and iron, HIF-1 alpha has one of the shortest half-lives of any known protein (28). In contrast, under hypoxic conditions, the HIF-1 complex is stable, allowing for transcriptional activation and ultimate stimulation of proteins such as erythropoietin and vascular endothelial growth factor (63).

Moreover, when altitude natives, or even altitude sojourners return to sea level, there is a suppression of erythropoietin (13, 17, 23, 30, 34, 43), a prominent reduction in iron turnover and bone marrow production of erythroid cell lines (26, 39), and a marked decrease in red cell survival time (39). This increase in red cell destruction with suppression of EPO levels has been termed “neocytolysis” and has been observed under other conditions of a relative increase in oxygen content (2-4, 41, 42). Both the rapid ubiquitination and destruction of HIF-1 alpha, and neocytolysis (which may be its clinical manifestation), may compromise the ability of short duration, intermittent hypoxic exposures to induce a sustained increase in the red cell mass.

In conclusion, it appears from the available evidence that real or simulated altitude exposures have the potential to increase the red cell mass, maximal aerobic power, and endurance performance in competitive athletes. However the “dose” of altitude must be at least that equivalent to 2,000-2,500m, for the majority of the day (i.e., more than 12 hrs), lasting at least 3-4 weeks to stimulate a sustained adaptive response in the majority of athletes. It is likely that some athletes, based on genetic predisposition may well have a beneficial response to lower “doses”, and that some athletes may never respond positively, even to

higher doses which then may be limited by toxicity. Future research should be directed to understanding the biological underpinnings of this diversity and defining more explicitly the optimal combinations of duration, frequency, and intensity for both individual and groups of athletes. Finally, it should be emphasized that the majority of this discussion is primarily relevant to well trained athletes who are using altitude exposure for discrete periods of time to boost sea level performance. There is much less information regarding longer durations of exposures (months to years) on athletic performance at sea level, or of the effects of these types of altitude exposures on performance at altitude. Both of these areas would be fruitful for further investigation.

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Chapter 21

THE EYE AT ALTITUDE

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Abstract High altitude retinopathy (HAR) was first described in 1969 as engorgement of retinal veins with occasional papilloedema and vitreous hemorrhage. Since then various studies have attempted to define the incidence, etiology and significance of this phenomenon, usually with small numbers of subjects. Recently studies on relatively large groups of subjects in Nepal, Bolivia and Tibet have confirmed that the retinal vasculature becomes engorged and tortuous in all lowlanders ascending above 2500m. Sometimes this leads to hemorrhages, cotton wool spots and papilloedema, which is the pathological state better known as high altitude retinopathy. These studies have also shown a significant change in both corneal thickness and intraocular pressure at altitude. The retinal blood vessels are the only directly observable vascular system in the human body and also supply some of the most oxygen-demanding tissue, the photoreceptors of the retina. New techniques are being applied in both hypobaric chamber and field expeditions to observe changes in retinal function during conditions of hypobaric hypoxia. This work allows better advice to be given to lowlanders traveling to altitude either if they have pre-existing ocular conditions or if they suffer from visual problems whilst at altitude. This especially applies to the effect of altitude on refractive eye surgery and results of recent studies will be discussed so that physicians can advise their patients using the latest evidence. Retinal hypoxia at sea level accounts for the developed world's largest cause of blindness, diabetic retinopathy. The investigation of retinal response to hypobaric hypoxia in healthy subjects may open new avenues for treatment of this debilitating disease.

Key Words: altitude, high altitude retinopathy, corneal pachymetry, intraocular pressure, refractive surgery

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INTRODUCTION

The eye, like every other organ, is affected by the hypobaric hypoxia of high altitude. This can cause decreased vision which can be life-threatening in a mountain environment, and is of interest to the clinician treating hypoxic eye disease at sea level.

There have been no previous measurements of corneal thickness at altitude in man; studies of intraocular pressure (IOP) have been hampered by small numbers of subjects and hostile conditions. High altitude retinopathy (HAR) was first recognized in 1969(71) but since then many studies have tried to assess its incidence and clinical significance with little success.

The preliminary results of ocular studies carried out on three large medical expeditions are discussed in this paper, the Medex expedition to Chamlang base camp in Nepal, the Apex 2 expedition to Chacaltaya in Bolivia and the Irvine Lovett Everest Expedition to the north side of Mount Everest in Tibet. These research expeditions had large numbers of subjects and different ascent profiles; as such they provided an excellent model for studying the effect of altitude on the eye.

An excellent review is available of the risks associated with ascent to altitude with pre-existing ocular conditions, so this will not be covered.(46) However the popularity of refractive surgery amongst high altitude mountaineers warrants discussion, as this has opened up new potential visual problems.

INTRAOCULAR PRESSURE

Intraocular pressure (IOP) at high altitude has been the subject of controversy for many years. In 1918 Wilmer and Berens measured IOP in 14 aviators in a hypobaric chamber but came to no significant conclusion.(83) More recently some groups have found decreased IOP,(10) whereas others have found increased IOP,(15) and normal IOP.(5, 17) Butler et al reported a significant relationship between IOP and flame hemorrhages associated with high altitude retinopathy(14) and one previous study showed a reduction in IOP that occurred within hours of ascent and recovered during acclimatization.(18)

IOP is determined by the rate of aqueous secretion, the resistance encountered in outflow channels and the level of episcleral venous pressure. The distribution of IOP within the normal population has a range of 11mmHg to 21mmHg (mean 16±2.5mmHg).(37) However IOP varies with the time of day, heart beat, blood pressure and respiration. The mean range of diurnal IOP fluctuations in normal eyes is 5mmHg with a tendency to be higher in the morning and lower in the afternoon and evening. It is therefore important to standardize the time of day when IOP readings are taken.

Approximately 80% of aqueous is produced by the non-pigmented ciliary epithelium as a result of an active metabolic process.(37) Therefore aqueous secretion should be diminished by factors that inhibit active metabolism such as hypoxia and hypothermia. Aqueous drainage is also partially an active process. There is no data on IOP changes in other chronic hypoxic conditions at sea level, such as chronic obstructive pulmonary disease and congenital cyanotic heart disease, and subsequently it is not known at what arterial oxygen saturation the autoregulatory control of IOP fails.

During the Apex 2 expedition, the opportunity arose to measure the IOP of 104 healthy

young lowlanders all ascending to 5200m with minimal exertion using the same ascent profile.

Materials and Methods

104 healthy subjects (56 male and 48 female) with a median age of 21 years (range 18-60) were selected to join the Apex 2 research expedition. At a pre-expedition meeting, subjects were consented, screened and sea-level measurements were recorded. There was no history of prior ocular disease and 18 subjects wore contact lenses. Subjects were then flown to La Paz (3700m) in Bolivia where they acclimatized for 4 days before being driven over 2 hours to the Cosmic Physics Laboratory at Chacaltaya (5200m). Testing took place on the first, third and seventh day in the laboratory. After the seventh day, the subjects were driven back down to La Paz. A post-expedition measurement was taken when at least one month after all subjects had returned to sea level. The ascent profile is shown in Figure 1.

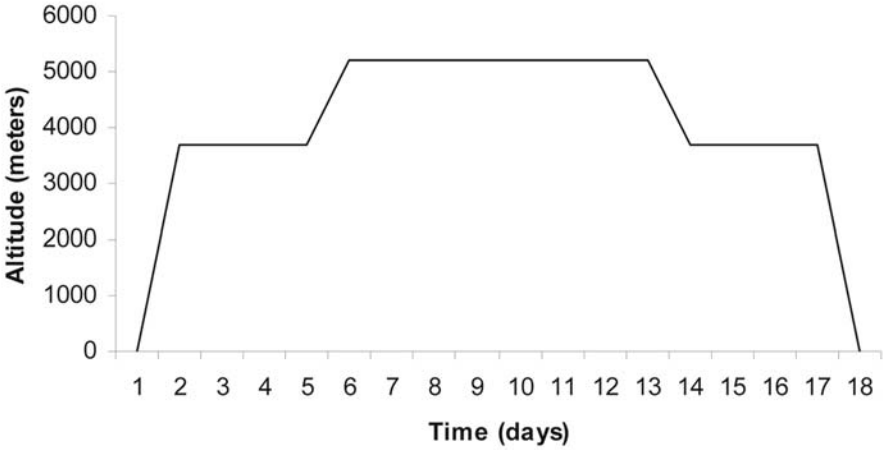

Figure 1. Apex 2 ascent profile.

In La Paz (3700m), subjects were advised to avoid any strenuous activity and were not allowed to descend below 2000m. In the laboratory (5200m), subjects were not formally restricted in their activity but were advised to rest for the first 2 days. After this they were allowed to exercise moderately if they wished but were not allowed to descend below 5000m. Cigarette smoking and alcohol consumption were prohibited for the duration of the experiment. AMS scores were recorded using the Lake Louise scale(66)

The subjects were also part of a double blind randomized controlled trial to compare the effect of sildenafil, anti-oxidants and placebo (see separate report) so use of medication was restricted to their experimental drug only, unless one of the expedition doctors had given permission. The effect of these trial drugs is discussed in the results section.

Choice of instrument for measuring IOP at altitude is difficult. Goldmann tonometry would be ideal but unfortunately requires a bulky slit lamp and is often poorly tolerated by young people. The Perkins tonometer is less accurate and difficult to calibrate. The air-puff tonometer has not been evaluated at altitude and is heavy and it is likely that the decrease in barometric air pressure would affect results.

In contrast the Tono-Pen is light, easy to calibrate and uses a pressure transducer. It is also well tolerated and has been proven in several trials to be comparable to the Goldmann applanation tonometer, especially in the normal range of intraocular pressure.(3, 24, 53) The Tono-Pen has a tendency to underestimate IOP above 30mmHg and overestimate IOP under 9mmHg.(38) However most readings at altitude are likely to be within the normal range of 11-21mmHg. The Tono-Pen is certainly the instrument of choice for field trials.(4)

IOP was measured for each eye with the Tono-Pen XL instrument (Figure 2). Each day the Tono-pen was calibrated to ensure accuracy and measurements were always taken in the early afternoon to avoid any diurnal variation. The intraocular pressure was measured 3 times for each eye after topical anesthesia with amethocaine. Visual acuity was measured at 6 meters under standard lighting conditions with a LogMAR chart.

Figure 2. Measuring IOP with the Tono-Pen XL (A Simpson)

Results

96 subjects (92 %) consented to take part in the eye study, 82 (78 %) consenting to the invasive testing of IOP measurement. Of these 89 (93 %) completed the first altitude testing session, 80 (83 %) the second and 73 (76 %) all three. The 23 (24 %) drop outs from the study were due to acute mountain sickness (AMS) in 17 cases and gastroenteritis and other conditions in the remaining 6 cases.

Analysis of the 3 sub-groups in the drug trial showed that neither sildenafil or the antioxidant preparation had any effect on the change in IOP. None of the subjects were allowed to take acetazolamide which is used as an IOP-lowering agent. There was also no statistical change in visual acuity from sea level.

The average IOP measurements are shown in Figure 3. A repeated ANOVA test was carried out on these results. Mauchly's test of sphericity was non significant (Chi-Square=10.1, df=5, p=0.07) but the multivariate F ratio was significant (F=3.07, df = 3, p=.037). Follow up paired comparison tests showed a significant difference between sea level and the first day at 5200m (p=0.006), borderline significance between sea level and the third day at 5200m (p=0.067) and no significant difference between the seventh day at 5200m and sea level (p=0.680).

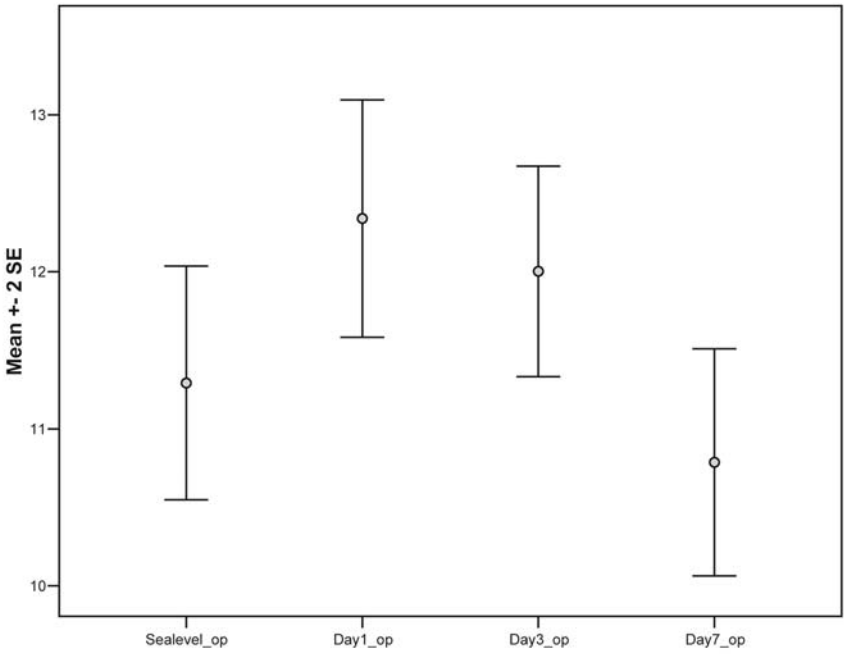

Figure 3. Change in intraocular pressure at altitude. There is an increase on the first day at 5200m which is significant, but IOP returns to sea level values by the seventh day at 5200m.

Discussion

Previous confusion over IOP at altitude may stem from poorly designed studies, small numbers of subjects, change in corneal thickness or because of a genuine change in IOP during acclimatization. Exercise has also been shown to reduce IOP as part of a hypotensive and sympathetic response.(44) Exercise conditioning can also significantly reduce baseline intraocular pressure.(61) This may be another confounding factor as subjects at altitude are often exercising more than normal and become increasingly fit during a sojourn to high altitude.

In contrast this study had a relatively large number of subjects and was rigorously designed as part of the Apex 2 expedition. Exertion was avoided as subjects were driven to 5200m. The results showed a significant increase in IOP on the first day at 5200m, which was less but still significant at the third day at altitude but had returned to near sea level

values by the seventh day.

These results do not fit with any previous investigation but may explain some of the past confusion. It appears that IOP rises on arrival at altitude but soon returns to normal. Therefore the timing of previous studies would have been crucial, as those testing IOP immediately on arrival at altitude would find an increase in IOP whereas those testing after the third day would find normal IOP or even decreased IOP because of the exertional factor.

This study used a fast ascent profile, designed to provoke altitude pathology. Normal ascent profiles may not identify this change in IOP as the IOP regulating mechanisms appear to acclimatize at the same time as the rest of the body.

Why is there an increase in IOP on arrival at altitude? As explained above IOP is determined by the rate of aqueous secretion, the resistance encountered in outflow channels and the level of episcleral venous pressure. It is unlikely the rate of aqueous secretion would increase at altitude as it is governed by active transport mechanisms that would be affected by hypoxia making a decrease in secretion more likely. The resistance in the outflow channels is also unlikely to change at altitude. However the episcleral venous pressure may increase as vessels dilate to cope with the decrease in oxygen saturation; this is the most likely cause of a transient rise in IOP during acclimatization.

This rise in IOP is unlikely to have an effect on the retinal circulation as the IOP still remains within normal limits, but further analysis is being carried out on optic disc photographs and frequency doubled perimetry that was carried out at 5200m to assess the impact of this IOP change on retinal function.

THE CORNEA

Corneal swelling due to hypoxia is a well known phenomenon, especially in relation to contact lens wear, but changes in corneal thickness due to the hypobaric hypoxia of altitude are less well described. Early studies showed that the normal human cornea will swell by 7% every hour in an oxygen-free environment.(63) Diurnal variation of central corneal thickness has also been described, with swelling up to 3.9% overnight, probably due to the hypoxia of lid closure; this swelling resolved by early afternoon.(74)

Corneal swelling is not just related to oxygen tension. Endothelial function and corneal metabolic activity are also important and various studies have attempted to determine the importance of each of these factors.(9, 59) For example it has been shown that contact lens wearers, whose corneas are in a constant state of mild hypoxia, do not adapt by modifying oxygen consumption but by using glucose more efficiently.(9) This raises the possibility that the cornea may be able to acclimatize to low atmospheric oxygen at altitude and that contact lens wearers may be less affected by altitude.

Diabetic subjects are more vulnerable to corneal hypoxia and have a reduced ability to recover after transient hypoxic edema, possibly due to enzymatic dysfunction of the endothelial pumps and a shift towards anaerobic metabolism.(84) However there are no reports of this affecting visual function outside the laboratory setting.

Previous studies have indicated that the physiological response of the cornea to soft contact lens wear at altitude are subject to higher levels of manifested stresses, but these occurred without measurable degradation in vision and do not preclude normal wear of soft contact lenses.(21) Rigid contact lenses tend to cause more corneal swelling than soft con-

tact lenses.(6) The aging cornea has a slower hypoxia-induced edema response compared with the younger group; whether this is caused by a decreased corneal lactate production or an increased resistance to physical expansion is unknown.(16, 62) One case study described a 77-year-old patient with low corneal endothelial cell counts who sustained acute unilateral endothelial decompensation when he traveled to an elevation of 12,500 feet. The corneal edema gradually increased after his return to sea level, and penetrating keratoplasty was required to restore vision.(40)

Once again, the Apex 2 expedition provided an excellent setting for measuring central corneal thickness in a large group of healthy lowlanders exposed to acute altitude.

Materials and methods

The subjects and experimental setting are as described previously, the only difference being that contact lens wearers were excluded from this study.

There are several methods available for measuring corneal thickness, such as specular microscopy, optical pachymetry, Orbscan Slitscan pachymetry and ultrasound pachymetry. Studies have shown that each technique delivers slightly different results, but the reliability of each individual technique is good.(49, 54) The ultrasound pachymeter is portable, reliable and depends on direct contact perpendicular to the cornea. It appears that central corneal thickness by ultrasound pachymetry can be adequately assessed in the majority of eyes by taking three measurements per eye.(64)

Corneal thickness was therefore measured with the Tomey SP-2000 ultrasound pachymeter (kindly loaned by the Princess Alexandra Eye Pavilion in Edinburgh). Measurements were taken 9 times for each eye after topical anesthesia with amethocaine (Figure 4). Corneal pachymetry was performed pre and post expedition and on the first, third and seventh day at 5200m, always early afternoon to avoid diurnal variation.

Figure 4. Measuring corneal thickness with ultrasound pachymetry (A Simpson)

Results

Analysis of the 3 sub-groups in the drug trial showed that neither sildenafil or the anti-oxidant preparation had any effect on the change in corneal thickness. As before there was no change in visual acuity.

The average corneal thickness measurements are shown in Figure 5 and a repeated ANOVA test was undertaken on the results. As Mauchly's test of sphericity was significant (Chi-Square=1, df=4, $p<0.001$) a multivariate analysis was used. This showed that the comparison between the two sea level values was not significant ($p=0.45$) but that all other altitude measures are highly significantly different from sea level ($p<0.001$). The pattern of the change in corneal thickness at altitude is shown in Figure 1. In addition to a significant increase in corneal thickness at altitude compared to sea level, there is also an apparent increase in corneal thickness with time spent at altitude. This was also found to be significant ($p=0.01$).

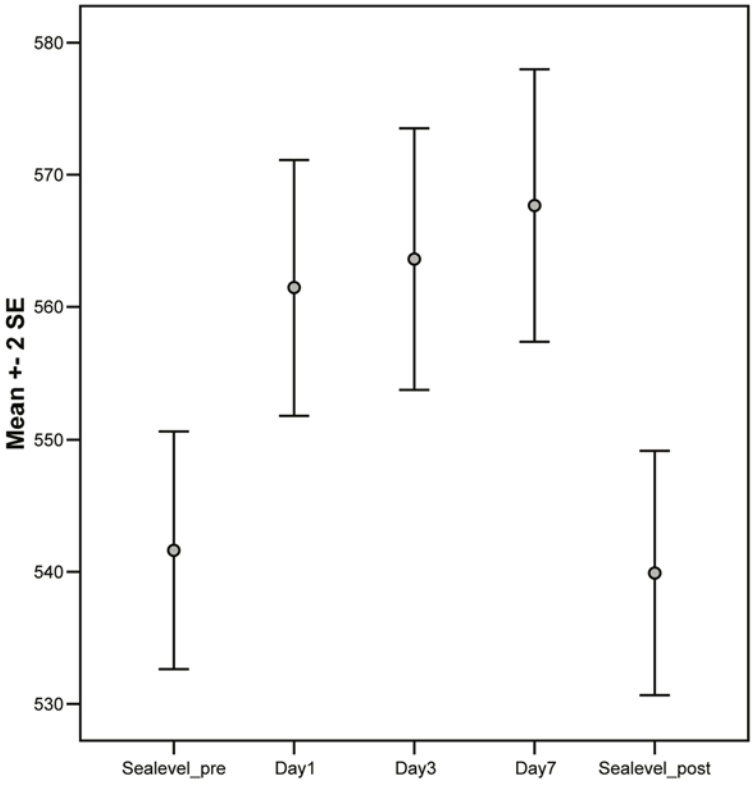

Figure 5. Change in corneal thickness at altitude. There is a clear difference between the sea level and altitude values and also an increase with time spent at altitude, both of which are significant.

DISCUSSION

For the first time there is evidence that hypobaric hypoxia causes a significant increase in corneal thickness in healthy lowlanders. This is significant from the point of view of IOP measurement and refractive surgery, as explained below.

There was marked variation between individuals dispute the majority being young and all contact lens wearers being excluded. There is evidence of individual susceptibility to corneal hypoxia; one study using a closed contact lens system to simulate a hypoxic environment over 3 hours.(68) The results showed a large variation in the amount of increased corneal thickness. This could be an indicator of the individual's capability to deal with hypoxia and acclimatize.

Which part of the cornea expands? Ultrastructural analysis of the rat cornea after exposure to a simulated altitude of 5,500 m for 30 days showed no change in the corneal epithelium.(50) This has been corroborated by evidence using optical coherence topography to measure corneal thickness after 3 hours of closed-eye contact lens wear which also showed no increase in epithelial thickness.(76) However the corneal stroma showed vascularization with advanced vessel differentiation and signs of active proliferation. The endothelium of hypoxic cornea showed swollen mitochondria and large empty cytoplasmic areas, and the endothelial intercellular junctions could hardly be identified in the hypoxic condition. Descemet's membrane was considerably thickened to approximately twice that of the control specimen. It therefore appears that the active transport mechanisms in the endothelium of the cornea, which normally maintain the cornea in its dehydrated state, are affected by hypoxia. This allows water to collect in the stroma causing the thickening seen in this experiment. Increased expression of both corneal epithelial vascular endothelial growth factor (VEGF) and cytochrome p450 4B1 was found in a rabbit model of closed eye contact lens wear, suggesting further evidence for the basic mechanism of hypoxic corneal swelling.(51)

It is interesting to compare these results with a group of similar subjects at sea level. A recent study of 1000 young, healthy, emmetropic eyes showed a range of 518-589um for central corneal thickness.(67) At altitude, the average corneal thickness did not exceed this normal range, explaining why there was no change in visual acuity. However within this range the average changed considerably from sea level to 5200m.

The cornea is dependent on aqueous humor for nourishment and the Fick equation shows that IOP measurement is dependent on corneal thickness.(37) If corneal thickness changes at altitude, then measured IOP changes may be artefactual. Previous studies have shown that a thickened cornea will give artificially high IOP readings and a thin cornea (for example following from excimer laser surgery(25)) will cause artificially low IOP readings.(13,36,77) However a recent study has cast doubt on these results by measuring central corneal thickness, IOP using applanation tonometry and IOP directly with an intracameral probe.(20) This showed no systematic error of applanation tonometry with corneal thickness. Regardless of these new findings, the corneal thickness and IOP are closely related and both need to be measured to accurately assess change at high altitude; in this case as the cornea thickened at altitude, the IOP decreased, suggesting that this was a real effect.

REFRACTIVE SURGERY AT ALTITUDE

The cornea is the main refractive structure in the eye. It is has been possible for some time to weaken or strengthen the power of the cornea surgically to avoid the use of spectacles and contact lenses. This type of refractive surgery has therefore become popular with active people who enjoy sports and outdoor recreation. However there are risks associated with the procedures, as with any surgery, and altitude can transiently affect the visual outcome. This could be life-threatening, as was demonstrated by the case of Dr Beck Weathers on Mount Everest in 1996. He had radial keratotomy and his vision became so blurred that he was unable to navigate back to his tent, losing his hands and nearly his life.

Radial Keratotomy (RK) was pioneered by the Russians in the 1980's. Deep radial incisions are made in the cornea to weaken and therefore flatten the surface. This makes the eye more hyperopic (long-sighted). Results were generally very encouraging, although it was difficult to predict exactly the final outcome, results were not always stable and occasionally the incisions were made too deep causing corneal perforation. Some climbers who had received RK noticed their vision became blurred at altitude and this was first reported in 1993 when a climber who had been under-corrected during RK noticed an improvement in his vision at 10,000 feet. (78) Refraction showed that he had become less myopic and therefore closer to his target refraction. The same authors investigated this phenomenon further trying to quantify and explain this change in refraction at altitude, using a variety of techniques including hypobaric chambers and sealed goggles to create a controlled environment and to separate the hypoxic and hypobaric elements of altitude which could potentially be causing this problem.(45,47,48,58,82)

They found a general trend towards hyperopia in subjects at altitude who had RK. This appears to be related to the decreased atmospheric pressure causing the weakened cornea to expand circumferentially. However there was a delay in the onset of refractive change at altitude so they hypothesized that this was not just a mechanical pressure effect, but there was also hypoxic expansion of the cornea around the radial incisions where there was sometimes endothelial cell loss.(7)

RK has now been superceded by excimer laser keratectomy, where a laser beam has replaced the traditional blade to shave layers off the cornea in a controlled, highly accurate fashion. There are two main techniques in use at present, laser in-situ keratomileusis (LASIK) and photo-refractive keratectomy (PRK). During the LASIK procedure a flap is made on the front of the cornea with a microkeratome and the excimer laser is then applied to remove a pre-determined amount of the corneal stroma. Once the flap is replaced, there is immediate, almost pain-free, visual recovery. However, there are occasionally flap-related complications, such as infection. During PRK, the corneal epithelium is removed and the laser applied directly to the cornea. However, there is a slight delay in visual recovery as the epithelium heals and it is more painful.

As both the lasers and the software controlling them becomes more sophisticated, there is now the possibility of super-vision, where the procedure can essentially remove all the tiny imperfections in the cornea as well as correcting the refractive error. This is obviously extremely attractive to pilots and those involved in outdoor pursuits.

Despite being superior to RK, LASIK and PRK do change the inherent structure and strength of the cornea, so the hypobaria and hypoxia of altitude can affect the refractive error. This was first reported in 2000 when a climber who had received LASIK experi-

enced blurred vision at 19,500 feet which cleared on descent.(79) Refraction showed this to be a myopic (short-sighted) shift, the opposite of the hyperopic shift from RK and this was confirmed by other case reports.(8) Mechanically, the anterior fibers of the cornea are cut when creating the LASIK flap, thus allowing the cornea to steepen at altitude, as opposed to the circumferential fibers that are severed during RK, causing corneal flattening. Sealed goggle experiments showed that hypoxia caused a slight steepening of the cornea in LASIK-treated eyes that was not statistically significant.(56)

The most impressive experiment to date involved six climbers on Mount Everest who all had LASIK.(19) Four reached the summit and all spent at least 24 hours above 26,400 feet. Although less people ascend to these extreme altitudes, it is here that altered vision could be potentially fatal. Three of the subjects had visual problems, one at 16,000 feet which cleared with acclimatization, one at 26,400 feet which made his vision hazy but did not stop him reaching the summit and finally one who turned around at 27,000 feet because of blurred vision.

The LASIK altitude myopic shift appears to be less common and less pronounced than the RK altitude hyperopic shift, which is logical as the procedure is less invasive. There is also an element of individual susceptibility as many people who have had refractive surgery experience no problems whatsoever, and this is corroborated by results in this paper on corneal thickness at altitude.

Physicians should be aware of these potential changes in patients who have had refractive surgery and intend to go to high altitude. As the results of refractive surgery can take some months to stabilize, patients should be advised not to receive treatment within 3 months of leaving on an expedition. Eyes treated with any refractive surgery are more susceptible to both dryness and infection, so patients and expedition doctors should be equipped to deal with these potential sight-threatening hazards.

HIGH ALTITUDE RETINOPATHY

In 1969, Singh et al published their observations of 1,925 Indian soldiers at high altitude. (71) 17 subjects complained of blurred vision (0.009%); on examination they noted engorgement of retinal veins in all 17 cases with papilloedema in 4 cases and vitreous hemorrhage in 3 cases.

Although Singh et al(71) are credited with the first description of HAR, Wilmer and Berens published a paper in 1918 on the effect of altitude on ocular functions.(83) They mention that Dr Guilbert of the French Air Service noted that "…at 2000m, in general, the visual acuity decreases by a third by reason of the congestion of all the organs of the head, and in particular of the choroid and retina." This is the first mention of retinal vascular engorgement at altitude in the literature.

It also appears that two other groups in North America discovered HAR at the same time as Singh et al but were slower to publish their findings. Frayser, Houston et al observed HAR on Mount Logan in 1969(22) as did Schumacher and Petajan on Mount McKinley in 1968 but did not publish their results until 1975.(69)

High altitude retinopathy (HAR) is a pathological response by the retina to the hypoxia of altitude (Figure 6). Flame hemorrhages are most commonly seen but cotton wool spots, dot and blot and pre-retinal hemorrhages have also been reported.(27) This should be dis-

tinguished from the normal physiological retinal response to the hypoxia of high altitude which includes retinal vascular tortuosity and engorgement and optic disc hyperemia.

Although HAR is usually asymptomatic, when a hemorrhage occurs over the macula, vision can be affected. This is occasionally reported in ocular studies at altitude as above but more often as individual case reports in the literature.(33, 73, 80) Macular involvement may be rare because there the area is avascular or because local factors ensure that nutrient supply to the macula is preserved in the hypoxic environment. Hemorrhages usually resolve without sequelae, but persistent field defects have been reported.(34, 70)

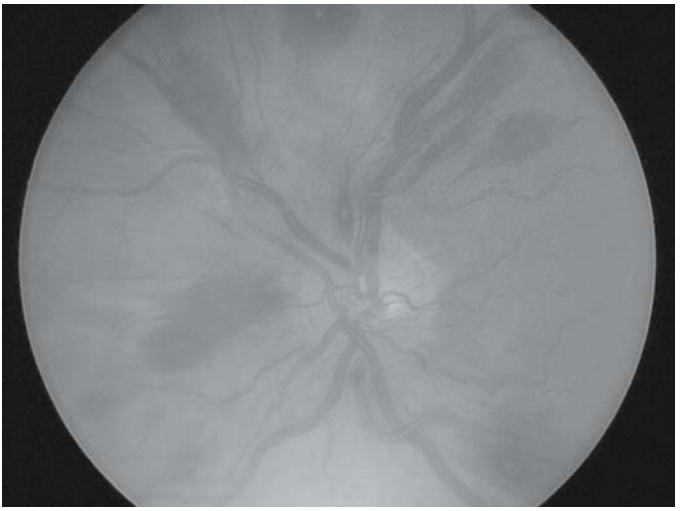

Figure 6. HAR - several flame hemorrhages and optic disk hyperemia.

Incidence of HAR

Table 1 shows the varying incidence of HAR described in the literature. The large range of incidence, from 3.8% to 90.5 %, reflects many different studies but may also provide a clue as to the pathophysiology of HAR. The maximum altitude attained appears to be a risk factor, and the low incidence of HAR in hypobaric chambers suggests that exertion may also be important.(11, 39, 72)

Digital fundal photography was performed before, during and after the 3 different expeditions mentioned earlier (Figure 7). HAR was defined as one or more hemorrhages in either eye and the incidences in table 2 below were recorded.

All subjects had their visual acuity assessed using a LogMAR chart but this was unaffected by altitude. AMS scores were recorded using the Lake Louise scale(66) and this data is still undergoing analysis. Incidence of HAR ranged from 10% to 64.2% but the maximum altitude attained did not appear to be significant as the Everest summiteers had the lowest incidence of HAR. This contradicts previous work,(81) but these mountaineers used supplemental oxygen above 7400m and when their overall ascent rate is examined, it was far lower than those who went to the north col of Everest, and who subsequently had the highest incidence of HAR (Figure 8). This raises the possibility that acclimatization is

important in preventing HAR. However, one would then expect the Apex 2 subjects to have a high rate of HAR with their extremely fast rate of ascent. But this was not the case, and the only difference was that these subjects were driven up to 5200m with no exertion. So these results point to a combination of poor acclimatization and exertion being the key risk factors in the development of HAR.

Table 1. The incidence of HAR in the literature.

Year	1st Author	Subjects	Max altitude	HAR (%)
1970	Frayser(22)	25	2959	36
1975	Schumacher(69)	39	6194	36
1989	Brinchmann-Hansen(10)	23	4000-5850	13
1975	Rennie(65)	15	5227-8167	33
1975	Shults(70)	6	5366-6890	67
1976	Clarke(17)	20	6500-8848	30
1979	Hackett(26)	340	5545	3.8
1981	McFadden(52)	39	5360	56.4
1983	Kramar(41)	7	6798	28.8
1992	Butler(14)	14	5300-8182	29
1999	Wiedman(81)	40	<7620	73.6
			>7620	90.5
2000	Müllner-Eidenböck(55)	7	7500-8201	25

Table 2. Incidence of HAR on 3 different expeditions.

Expedition	Subjects	Max altitude (m)	Ascent rate (m/day)	HAR (%)
Everest summit	20	8848	140	10
Everest north col	14	7100	338	64.2
Medex 2003	52	6500	210	28.3

Pathophysiology of HAR

There have been many proposed theories for the development of HAR, but none of them have been thoroughly tested (table 3).

One hypothesis that has been recently tested is that of changed blood viscosity. Erythropoietin is released in response to the lowered arterial oxygen saturation at altitude which causes a rise in hematocrit.(35) This response varies considerably between individuals and the increased blood viscosity has been suggested as another mechanism for the develop-

ment of HAR.(42) The Medex expedition in 2003 provided the opportunity to test this hypothesis.

Volunteers were recruited and consented at a pre-expedition weekend. They then trekked from Tumlingtar (300m) to Chamlang base camp (5100m) in Nepal over 19 days following a safe acclimatization regime. All measurements were taken at sea level and at 5100m. Capillary hematocrit was assessed using a Clinispin Hct Benchtop Centrifuge; samples were spun at 3,000 rpm for 15 minutes and the proportion of packed red blood cells to plasma was measured immediately against a visual scale. Digital fundal photography of the posterior pole was carried out using a Nidek NM-100 camera. Subjects with co-existing ocular pathology and those who took acetazolamide were excluded.

Forty six subjects fit the criteria for the study. There were 25 males and 21 females with a mean age of 33.3 years, the oldest being 53 and the youngest being 21. Mean hematocrit at sea level was 40.4 % (SD 3.7). This increased to 48.1 % at 5100m (SD 4.7). A paired t-test showed this change to be highly significant ($p<0.05$). HAR, defined as one or more hemorrhages at 5100m in either eye, was found in 13 out of 46 subjects (28.3 %). None of these subjects reported any visual symptoms and the hemorrhages cleared within 2 weeks without sequelae. These subjects did not have any significantly greater or smaller change in hematocrit, as measured by the two-tailed Wilcoxon rank sum test ($p=0.66$).

The incidence of 28.3 % in this study would fit with a cohort ascending sensibly to moderately high altitude. However these results do not support the hypothesis that increased blood viscosity secondary to raised hematocrit at altitude is a causative factor in the development of HAR. In this study, hematocrit rose as expected but the extent of this increase was not predictive of HAR.

Figure 7. Digital retinal photography at Everest basecamp (5200 m).

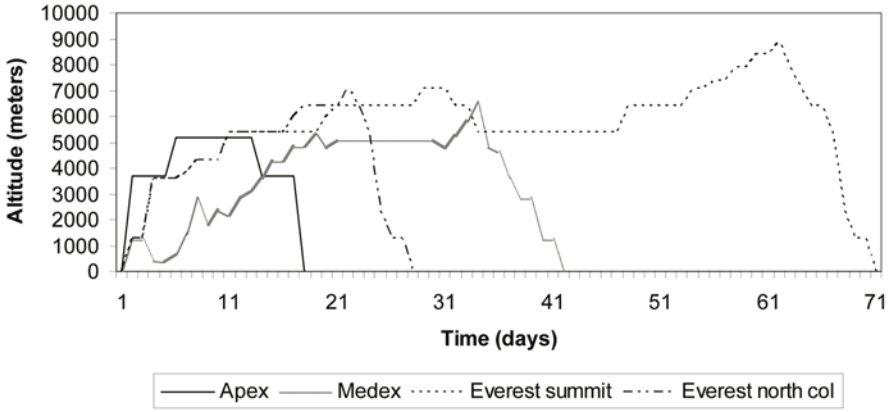

Figure 8. Different ascent profiles of each expedition; Apex 2 had the fastest rate of ascent and the Everest summiteers the slowest rate of ascent.

Retinal blood flow regulation

Regulation of blood flow to the retina and optic nerve remains constant over a range of elevated intraocular pressure or mean arterial pressure, independent of sympathetic activation (pressure autoregulation). In addition, increased metabolic activity in these tissues proportionally increases blood flow (metabolic autoregulation). At constant metabolic rate, altered arterial oxygen content reciprocally alters blood flow, leaving total oxygen delivery constant, while blood flow rises and falls with the arterial carbon dioxide tension. These responses are similar to those of the cerebral circulation.(30) In retinal circulation, systemic controls have only a minor influence, while local factors (e.g. nitric oxide, prostaglandins, endothelin, and the renin-angiotensin system(28)) dominate regulation.(60)

At sea level, the main factor for retinal vascular smooth muscle is the arterial carbon dioxide tension; blood flow varies directly with the PCO_2.(29) However at altitude there is evidence that hypoxia, rather than hypocapnia, becomes the controlling factor in retinal circulation.(23)

There is also an apparent change in retinal arterial response to exercise at simulated altitude after 7 weeks acclimatization; relative constriction to exercise was replaced with dilation. This provides a clue to a change in the autoregulatory pattern, possibly illustrating an increased state of readiness for exercise in the hypoxic state.(12)

With currently available evidence, it would appear that the pathophysiology of HAR is a two-stage process; firstly the hypoxic stress of altitude causes retinal vascular engorgement to maintain adequate oxygen supply for the metabolically-demanding photoreceptors; this is a normal physiological response. Secondly there is some overload of the vascular system, such as exertion, which pushes the regulatory system beyond its physiological limits, causing the pathological breakdown of the capillary endothelium and hemorrhage.

Table 3. Theories for the pathophysiology of HAR

- Increased ocular blood flow (80)
- Retinal venous outflow obstruction (22)
- Increased IOP (22)
- Valsalva retinopathy (65)(70)
- Increased blood pressure (22)
- Raised intracranial pressure (17,22,81)
- Vascular headache mechanism (69)
- Vascular dysregulation (55)
- Increased blood viscosity (42,65,70)
- Hypoxic vascular weakness (65)
- Decreased atmospheric ozone (70)
- Hypoxic endothelial decompensation (41,80)
- Platelet microemboli (34)

FUTURE DIRECTIONS

The clear optical media of the eye allow direct observation of a vascular bed at altitude and recent technological advances have allowed investigators to take advantage of this window. Müllner-Eidenböck et al recently used the Heidelberg retinal flowmeter in an altitude study.(55) This device uses laser Doppler flowmetry and laser scanning tomography to produce a reading for retinal blood volume, flow and velocity. Instruments such as this and the Paradigm ocular blood flowmeter, will provide a more dynamic picture of changes to the vasculature in the eye at altitude and may explain exactly what precipitates retinal hemorrhages. Magnetic resonance imaging has also been performed on 2 subjects who had HAR after ascending Aconcagua (6986m) which showed no significant changes.(43)

Vascular endothelial growth factor (VEGF) is an angiogenic protein and vasopermeability factor which is released at high altitude.(75) Intraocular VEGF concentrations are closely correlated with active neovascularization in patients with diabetes mellitus, central retinal vein occlusion, retinopathy of prematurity and rubeosis iridis.(1) In-vitro experiments have shown that hypoxia increases VEGF expression in retinal cells.(2) VEGF has also been found in hypoxic corneal epithelial cells as described above.(51) The role of VEGF in ocular altitude problems is not yet understood, but clearly warrants further research.

The optic nerve sheath is continuous with the dura mater and its diameter can be measured using ocular ultrasound. This has been shown to be a reliable non-invasive measure of intra-cranial pressure.(31, 32, 57) A recent pilot study in Colorado showed a small increase in optic nerve sheath diameter after brief decompression to 14,000 feet. This will be further investigated and may prove important in the early diagnosis of HACE.

Visual acuity is a coarse measure of retinal function and therefore rarely changes during studies at altitude. More sensitive measurements include contrast sensitivity, luminance thresholds, dark adaptation, macular recovery time, visual fields and color vision. As more sophisticated tests emerge, they will be used in altitude experiments to asses the effect of altitude on retinal function, rather than just examining the retinal vasculature (Figure 10).

Most altitude research is carried out on lowlanders ascending for a short time to high altitude. There are a large number of people throughout the world who live above 2500m and studying the retinal circulation in these people may provide an insight into the effect of chronic hypoxia on the retina.

There is a need for hypothesis-driven research with larger numbers of subjects on research-orientated expeditions. This should be combined with controlled experiments in hypobaric chambers and perhaps the development of an animal model for HAR.

Figure 9. Measuring color vision with a prototype instrument at Everest north col (7100m).

CONCLUSION

These studies have begun to shed light on corneal and IOP changes at altitude. However it is now over 35 years since the original observation that retinal hemorrhages occur at high altitude and despite a considerable amount of work on the subject, the cause and possible clinical implications of HAR remain obscure. A better understanding of the pathophysiology of HAR may shed light on the basic mechanisms of hypoxic illness at sea level; diabetic retinopathy and retinopathy of prematurity are caused by retinal hypoxia.

There has been an attempt to classify HAR(81) but as most people observing this phenomenon are not ophthalmologists, it would be better to simplify the definition of HAR to

"one or more hemorrhages in either eye of a person ascending above 2500m" with secondary signs of decreased visual acuity, papilloedema and cotton wool spots. Retinal vascular engorgement and tortuosity should be viewed as the normal physiological response to hypoxia.

HAR is not confined to the realms of extreme altitude and every year more people venture above 2500m for recreation, for example Honigman et al recently described a case of HAR in a Colorado skier.(33) Any change in vision at altitude warrants descent, as the cause is not always obvious as it could be a benign change in the cornea after refractive surgery or a potentially fatal episode of cerebral hypoxia.

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ABBREVIATIONS

AMS - Acute mountain sickness
BP - Blood pressure
CCAMP - Colorado Centre for Altitude Medicine and Physiology
CSF - Cerebrospinal fluid
HAR – High altitude retinopathy
HAPE – High altitude pulmonary edema
HACE – High altitude cerebral edema
ICP - Intracranial pressure
IOP - Intraocular pressure
LASIK - Laser in-situ keratomileusis
NSAID - Non-steroidal anti-inflammatory drugs
PRK - Photo-refractive keratectomy
RK – Radial Keratotomy
REDIARC - Retinal Diseases Image Analysis Reading Center
VEGF – Vascular endothelial growth factor.

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Chapter 22

LAKE LOUISE CONSENSUS METHODS FOR MEASURING THE HYPOXIC VENTILATORY RESPONSE

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BACKGROUND

The hypoxic ventilatory response (HVR) is the body's first line of defense against environmental hypoxia, for example, at high altitude. In acute hypoxia, the HVR is a reflex increase in ventilation in response to decreased arterial PO_2, which is rapidly sensed by arterial chemoreceptors primarily in the carotid bodies.With sustained exposure to hypoxia, the HVR changes (8). For example, after 5 to 20 minutes of hypoxia, ventilation "rolls off" relative to the acute hypoxic response and this is called hypoxic ventilatory decline (HVD). With continuous hypoxia, for example during acclimatization to high altitude, ventilation increases above the level of the acute HVR with ventilatory acclimatization to hypoxia (VAH). The different time domains of the HVR raise many questions about the physiological mechanisms of oxygen sensing and the control of breathing. They also raise many issues about experimental methods to measure the HVR and comparing results between laboratories. At the 13th International Hypoxia Symposium, John Severinghaus proposed consensus methods for measuring the HVR in humans, and the discussion continued at the 14th International Hypoxia Symposium in Lake Louise. The goal of this report is to stimulate further discussion and experiments so we can adopt a "Lake Louise Consensus for Measuring the HVR" at the 15th International Hypoxia Symposium in 2007.

METHODS AND RESULTS

An example of a study using methods that are similar to early drafts of a consensus for measuring the HVR in humans is found in an abstract in these proceedings (12). It uses steady-state measurements of the HVR so ventilation is measured as a function of constant

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level of hypoxic stimulation, in contrast to methods relying on continuously changing PaO_2 (13) or cyclic changes in PaO_2 (6). Breathing circuits for this kind of measurement are described in the literature (11).

Ventilation was measured from subjects wearing a face mask. PIO_2 and $PICO_2$ were adjusted to obtain desired levels of arterial O_2 saturation estimated with a pulse oximeter (SpO_2), and end-tidal PCO_2 ($PETCO_2$). Figure 1 illustrates the key points of the protocol to measure HVR and shows average results from Steinbeck and co-workers (12).

After acclimating to the apparatus while breathing room air (ca. 15 min), PIO_2 is rapidly decreased to produce a target SpO_2 of 80%, while $PICO_2$ is kept at 0 Torr, to measure the poikilocapnic HVR. This level of PIO_2 is maintained for 20 min. Because the change in V. E is so small with hypocapnia from the hypoxic hyperventilation, the poikilocapnic HVR is quantified as: $pHVR = \Delta PETCO_2 / \Delta\ SpO_2$. The pHVR5 measured after 5 min of poikilocapnic hypoxia is a measure of the acute poikilocapnic HVR before significant HVD occurs. The $pHVR_{20}$ measured after 20 min of hypoxia quantifies the poikilocapnic HVR in the presence of HVD.

Subjects then rested 1 hr before measuring the isocapnic HVR using a similar protocol. $PETCO_2$ was increased 1 Torr above the level measured during the first 5 min of mild hyperoxia and held at that level for another 10 min of hyperoxia. PIO_2 was decreased to give a target SpO_2 of 80% and $PICO_2$ was adjusted as necessary to maintain $PETCO_2$ throughout 20 min of sustained isocapnic hypoxia. The isocapnic HVR was quantified as: $iHVR = \Delta \dot{V}_E / \Delta SpO_2$. The $iHVR_5$ measured the acute isocapnic HVR before significant HVD and $iHVR_{20}$ measured the HVR after HVD had stabilized under isocapnic conditions.

In Steinbeck et al's. subjects, $iHVR_5 = 2.37 \pm 0.61$ (L/min%) and this decreased with HVD so $iHVR_{20} = 0.75 \pm 0.27$ (L/min%). These values are similar to other publications using similar methods and protocols (3, 10, 11). The effects of HVD on the measure of poikilocapnic HVR were much smaller: $pHVR_5 = 0.33 \pm 0.05$ (Torr/%) and $pHVR_{20} = 0.28 \pm 0.08$ (Torr/%).

DISCUSSION

The results presented above demonstrate a relatively simple and short protocol can generate reproducible measures of the HVR that are comparable between different laboratories. However, there are many questions that need further discussion and study before endorsing consensus methods. These are presented briefly below.

Steady state method

This approach is chosen because it is relatively simple and requires only the most basic respiratory physiology equipment. Additional information about the physiology of the various time domains of the HVR may be obtained by more sophisticated methods relying on rapid, cyclic changes in O_2 level. HVD may start decreasing ventilation within 2 min of sustained hypoxia (1) so $iHVR_5$ may not be a perfect measure of the acute HVR but it is robust. Rebreathing methods may offer an advantage when gases are not available to control steady PIO_2 and $PICO_2$ levels (e.g. at altitude) but the results are more difficult to interpret given with simultaneous time-dependent changes in the HVR and stimulus levels.

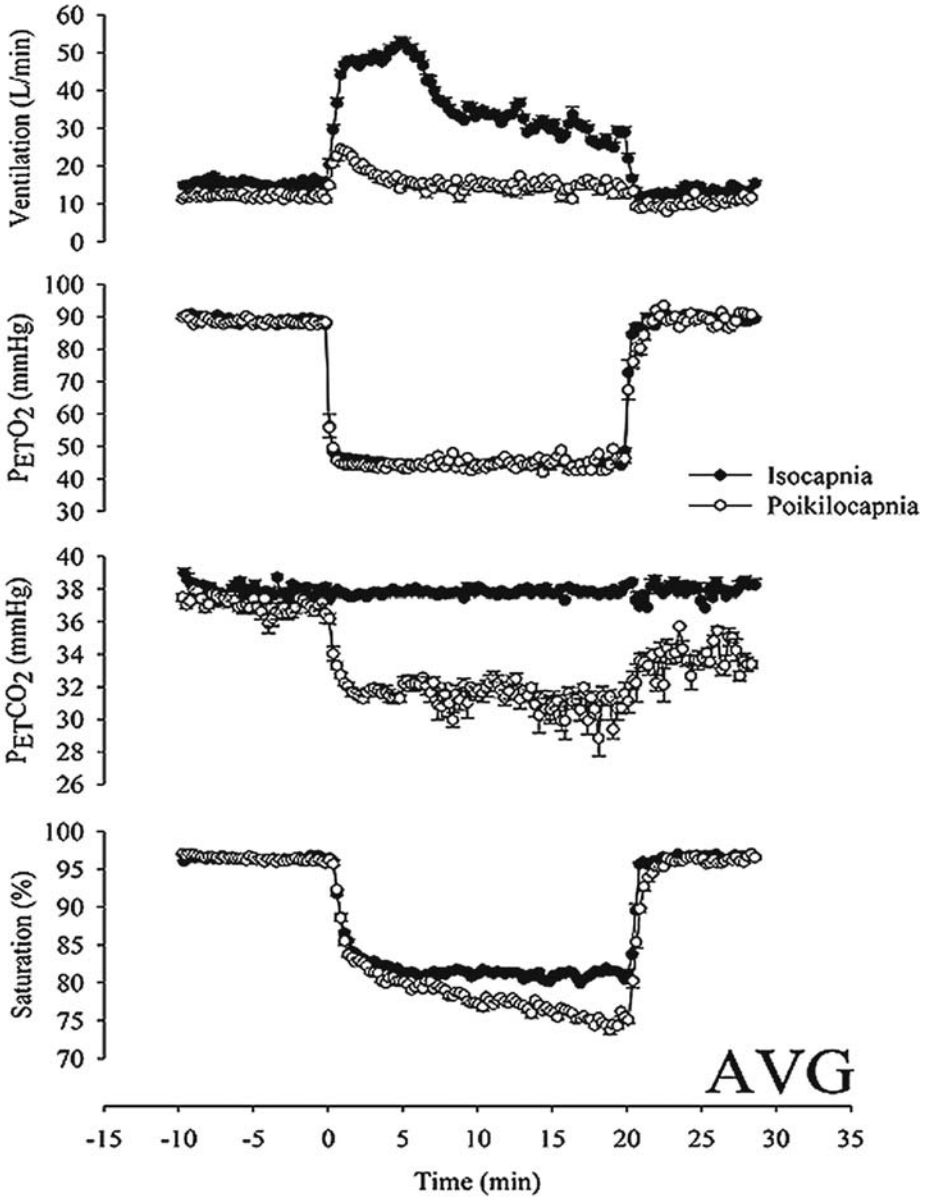

Figure 1. Average respiratory variables for normal humans (n=7) during normoxia, 20 min of hypoxia and normoxic recovery (12).

Ventilatory baseline

Most subjects show small decreases in $\dot{V}_E$ and increases in $PETCO_2$ breathing 30% O_2 versus room air (21%) at sea level. Hence, mild hyperoxia (PIO_2 = 200 Torr) has been used to minimize O_2-sensitive ventilatory drive from arterial chemoreceptors (11). However, animal studies indicate that even such mild hyperoxia may be a ventilatory stimulant and there is also some evidence for this in some human studies that should be evaluated further (cf. (2)). The ideal duration for increasing O_2 level to establish the ventilatory baseline needs to be determined. For subjects acclimatized to 3,800 m, additional time at high O_2 levels was necessary to remove underlying HVD with chronic hypoxia at altitude (11).

Hypoxic stimulus

The hypoxic stimulus to carotid body chemoreceptors is arterial PO_2 and not arterial O_2 content or saturation. However, $\dot{V}_E$ is a linear function of SaO_2 between 100% and 70% (9, 11) allowing the HVR to be expressed independent of the exact level of hypoxia within this range as $\Delta\dot{V}_E/\Delta SpO_2$. This is not meant to imply that the stimulus is SaO_2, however. This protocol held PIO_2 constant after achieving the initial target for hypoxic stimulation but other approaches include adjusting PIO_2 to maintain constant $PETO_2$ or SpO_2 during sustained hypoxia.

CO_2 level for isocapnia

Results appear similar in different studies that have maintained isocapnia either (1) 1-2 Torr above the level measured during normoxia (this study), (2) 1-2 Torr above the level measured during mild hyperoxia (3), or (3) at the level necessary to increase $\dot{V}_E$ with PIO_2 = 200 Torr to a target level(4, 11). However, choosing the $PETCO_2$ level for the isocapnic HVR during acclimatization to altitude is more difficult because the arterial CO_2 set point decreases as ventilatory drive increases with VAH. Efforts to maintain ventilatory drive constant at different time points during acclimatization are difficult to interpret because interactions between the HVR and other ventilatory drives being manipulated experimentally (e.g. central CO_2 drive, cf. (11)) could be changing during VAH.

Time points and measurements

The 5 min time point for measuring the HVR is a compromise between using simple methods to establish a constant stimulus level (SpO_2 = 80% and constant $PETCO_2$) and the desire to measure the HVR as soon as possible before significant HVD can occur. Sophisticated methods show some HVD occurs as soon as 2 min after the start of hypoxia but the changes are minimal compared to those measured between 5 and 20 min of hypoxia (1). Of course it is ideal if continuous measurements are possible but agreeing on specific time points is necessary for a consensus to compare between different experiments and investigators.

The order of measuring the isocapnic and poikilocapnic HVR should also be considered if they are studied in an individual on the same day. Steinbeck and co-workers found a tendency for $\dot{V}_E$ to remain elevated for more than 20 min following isocapnic hypoxia in some individuals, presumably because of lactic acidosis. Hence, it may be preferable to measure the poikilocapnic HVR before the isocapnic HVR if they must be measured sequentially on the same day.

Finally, the individual components of $\dot{V}_E$, i.e. respiratory frequency and tidal volume, should be measured and reported.

Other factors

Other factors that may influence the HVR and should be at least reported, if not standardized, include: posture (e.g. semi-recumbent 30° head up (11)), open versus closed eyes (affects resting $PETCO_2$, JW Severinghaus, personal communication), auditory stimuli (listening to music or watching a movie should not drive breathing), coaching by the investigators on how to breathe (should alleviate stress response and unmask reflex responses), a facemask or a mouthpiece (different effects on pattern of breathing in some subjects), the phase of the menstrual cycle or birth control pills in women (hormones affects the HVR), medications (e.g. aspirin enhances the HVR), fasting versus post-prandial state and wakefulness.

Physiological significance and interpretation

The goal of measuring the HVR is frequently to investigate a physiological mechanism of hypoxic sensitivity. For example, people reading this volume and attending the International Hypoxia Symposia may be interested in the physiological mechanisms of change in the HVR during ventilatory acclimatization to high altitude. However, the HVR is a reflex measurement, so it is a challenge to distinguish differences between O_2 sensing, integration within the central nervous system, and changes in respiratory motor output (7). Still, the effort to develop a consensus method for measuring the HVR should prove valuable even if it does not include standard physiological interpretation of the results. For example, the Lake Louise scoring system for AMS has been extremely useful for increasing our understanding of the physiology and pathology of acute mountain sickness (5).

FUTURE DIRECTIONS

The draft consensus methods for measuring HVR are available on the hypoxia web site: http://www.hypoxia.net. Interested parties are encouraged to access the web site and use the contact information found there to expand the discussion of this topic. An updated draft will be posted before the 15th International Hypoxia Symposium in 2007 with the goal of adopting a “Lake Louise consensus for measuring the HVR” at that meeting.

ACKNOWLEDGEMENTS

John Severinghaus is responsible for initiating the effort to develop a consensus method for measuring the hypoxic ventilatory response. Jerry Dempsey and Tom Hornbein stimulated many provocative discussions on this problem and continue to provide valuable guidance in efforts to understand the hypoxic ventilatory response.

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Chapter 23

PULMONARY HYPERTENSION IN HIGH-ALTITUDE DWELLERS: NOVEL MECHANISMS, UNSUSPECTED PREDISPOSING FACTORS

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Abstract: Studies of high-altitude populations, and in particular of maladapted subgroups, may provide important insight into underlying mechanisms involved in the pathogenesis of hypoxemia-related disease states in general. Over the past decade, studies involving short-term hypoxic exposure have greatly advanced our knowledge regarding underlying mechanisms and predisposing events of hypoxic pulmonary hypertension. Studies in high altitude pulmonary edema (HAPE)-prone subjects, a condition characterized by exaggerated hypoxic pulmonary hypertension, have provided evidence for the central role of pulmonary vascular endothelial and respiratory epithelial nitric oxide (NO) for pulmonary artery pressure homeostasis. More recently, it has been shown that pathological events during the perinatal period (possibly by impairing pulmonary NO synthesis), predispose to exaggerated hypoxic pulmonary hypertension later in life. In an attempt to translate some of this new knowledge to the understanding of underlying mechanisms and predisposing events of chronic hypoxic pulmonary hypertension, we have recently initiated a series of studies among high-risk subpopulations (experiments of nature) of high-altitude dwellers. These studies have allowed to identify novel risk factors and underlying mechanisms that may predispose to sustained hypoxic pulmonary hypertension. The aim of this article is to briefly review this new data, and demonstrate that insufficient NO synthesis/bioavailability, possibly related in part to augmented oxidative stress, may represent an important underlying mechanism predisposing to pulmonary hypertension in high-altitude dwellers.

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Key Words: re-entry pulmonary edema, preeclampsia, oxidative stress, trisomy 21

INTRODUCTION

High altitude constitutes an exciting natural laboratory for medical research. Over the past decade, the scope of high altitude research has broadened considerably, since it has become clear that besides of being of importance for the prevention and/or treatment of altitude related diseases in climbers, the results of this research also may have important implications for the treatment of patients at low altitude, and for the understanding of diseases in the millions of people living permanently at high altitude.

Our group, after having devoted a decade or so, to the study of short-term adaptation to high altitude in mountaineers, has recently started to translate some of this new knowledge gained during these studies, to the study of long-term adaptation in high-altitude dwellers. In this article, we will first briefly summarize some of the salient results gathered during the short-term high altitude studies (with particular focus on pulmonary artery pressure and mechanisms of pulmonary edema), and then demonstrate the potential importance of this new knowledge for the long-term adaptation of high-altitude dwellers.

SHORT-TERM ADAPTATION TO HYPOXIA

Mechanisms underlying exaggerated pulmonary vasoconstrictor responsiveness during short-term high altitude exposure

Over the past two decades, studies in subjects susceptible to high-altitude pulmonary edema (HAPE), a condition characterized by exaggerated hypoxia-induced pulmonary vasoconstriction, have provided important new insight into the regulation of pulmonary-artery pressure at high altitude.(41)

Nitric oxide

These studies have provided evidence for the importance of pulmonary vascular endothelial and alveolar epithelial nitric oxide (NO) synthesis in the regulation of pulmonary vascular responsiveness to high-altitude exposure. The evidence is as follows: NO plays an important role in the regulation of pulmonary vascular tone in humans, because inhibition of NO synthesis by L-NMMA infusion potentiates the pulmonary vasoconstrictor response evoked by short-term hypoxic breathing. (6) In certain populations, HAPE susceptibility has been found to be associated with eNOS polymorphisms and impaired vascular NO synthesis. (1, 15) NO, when administered by inhalation, lowers pulmonary-artery pressure to a much larger extent in HAPE-susceptible subjects than in control subjects who never experienced HAPE.(42) These observations suggest that defective pulmonary endothelial NO synthesis is one of the mechanisms contributing to exaggerated hypoxic pulmonary hypertension in humans. Parenthetically, it is interesting to note here that at physiological concentrations, NO attenuates oxidative stress, a mechanism that has been implicated in

the pathogenesis of hypoxic pulmonary hypertension. (7, 22, 25) In eNOS deficient states, loss of NO inhibition of oxidative stress may therefore represent an additional mechanism facilitating pulmonary hypertension.

In the respiratory system, NO is not only produced by the pulmonary vascular endothelium, but also by the respiratory epithelium, and there is evidence that the latter also regulates pulmonary artery pressure.(43) Respiratory epithelial, but not pulmonary endothelial, NO synthesis can be assessed by measuring NO in the exhaled air.(11, 37) In HAPE prone subjects, exhaled NO at high altitude is lower than in control subjects (Figure 1A), and there exists an inverse relationship between pulmonary-artery pressure and exhaled NO at high altitude (Figure 1B).(16) Taken together, these findings indicate that defective pulmonary endothelial and respiratory epithelial NO synthesis contributes to exaggerated pulmonary hypertension during short term high altitude exposure.

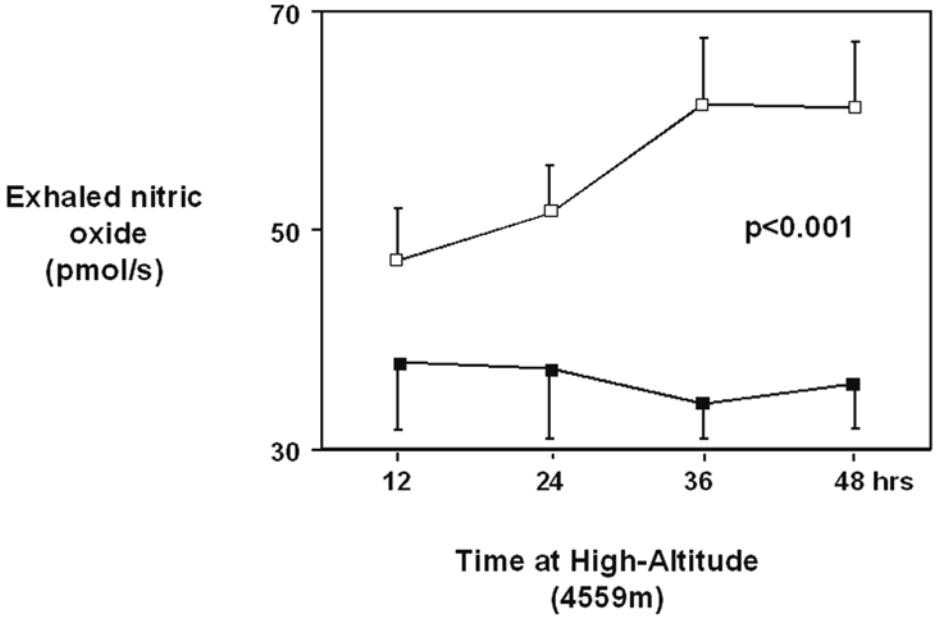

Figure 1A. Line graphs showing the effects of high-altitude exposure on exhaled pulmonary NO in HAPE-prone and resistant subjects. In the HAPE-prone subject, exhaled NO was significantly lower ($p<0.001$) than in HAPE-resistant subjects. (Adapted from Duplain et al. (16))

Endothelin-1

Recent evidence suggest that not only impaired pulmonary synthesis of the vasodilator NO, but also augmented production of the potent pulmonary vasoconstrictor ET-1, may contribute to exaggerated pulmonary vasoconstriction at high altitude. Indeed, it is well established that high-altitude exposure stimulates ET-1 synthesis in humans, and we found that there exists a direct relationship between the altitude-induced stimulation of ET-1 synthesis and the increase in pulmonary-artery pressure in humans.(40) Interestingly, defective pulmonary NO synthesis and augmented ET-1 synthesis could be causally related, because NO inhibits hypoxia-induced stimulation of ET-1 synthesis in human endothelial cells in vitro. (28)

Sympathetic nervous system

There is abundant evidence that cardiovascular adjustments to hypoxia are mediated, at least in part, by the sympathetic nervous system. (29) Studies in HAPE-susceptible humans indicate that hypoxia-induced sympathetic over-activity may represent an underlying mechanism for exaggerated pulmonary hypertension at high altitude. (17) Most interestingly, studies in experimental animals and humans indicate that central neural NO plays an important role in buffering sympathetic outflow.(32, 38) This observation could be consistent with the hypothesis that defective NO synthesis could lead to exaggerated pulmonary hypertension by the combination of loss of NO-induced vasodilatation, and facilitation of ET-1- and sympathetically-mediated vasoconstriction.

In conclusion, while these studies provided important insight into mechanisms involved into exaggerated pulmonary vasoconstriction during short-term high-altitude exposure in humans, the equally important issue of factors or events predisposing and/or protecting humans from exaggerated hypoxic pulmonary hypertension remained largely "terra incognita". Recent studies have started to shed some light on this issue.

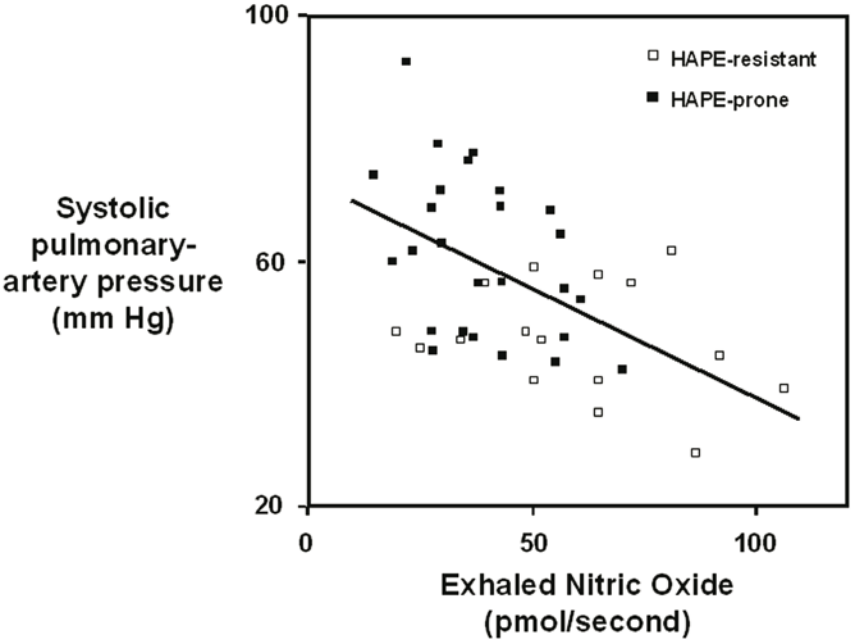

Figure 1B. Plot of the relationship between exhaled pulmonary NO and systolic pulmonary artery pressure at high altitude (4,559 m). r=-0.51, P<0.001. (Adapted from Duplain et al. (16))

Factors predisposing to exaggerated pulmonary hypertension during short-term hypoxic exposure

Perinatal hypoxia

Epidemiological studies suggest that adverse events in utero may predispose to cardiovascular and metabolic disease in adulthood. (2) In mammals and humans, at birth,

the transition from gas exchange by the placenta to gas exchange by the lungs, requires dramatic changes in the pulmonary blood vessels which during this period are particularly vulnerable to noxious stimuli such as hypoxia. (30) In rats, exposure to hypoxia during the first days of life induces a transient increase of pulmonary artery pressure, and predisposes to exaggerated pulmonary vasoconstrictor responses to hypoxia and monocrotaline in the adult life.(20) During studies at the high-altitude research laboratory Capanna Regina Margherita in the Alps (4559 m), we have demonstrated a similar phenomenon in young healthy adults who had suffered from transient lack of oxygen during the first few days after birth.(35; Figure 2) The mechanism underlying this exaggerated vasoconstrictor response is not known yet, but there is evidence that this pathologic response is related to a functional rather than a structural defect. Data in rats show that transient hypoxia during the first few days leads to decreased eNOS expression in the lungs. (45) Thus, impaired NO synthesis may represent a potential mechanism. In line with this hypothesis, NO inhalation caused a substantially larger decrease in pulmonary artery pressure in the subjects with a perinatal insult than in control subjects.(35)(Figure 2) These findings demonstrate that in humans, a transient insult to the pulmonary circulation during the perinatal period, leaves a persistent and potentially fatal imprint (possibly defective NO synthesis) which, when activated later in the life, predisposes to a pathological response. This observation suggests that survivors of perinatal pulmonary hypertension may be at risk of developing this disorder later in life.

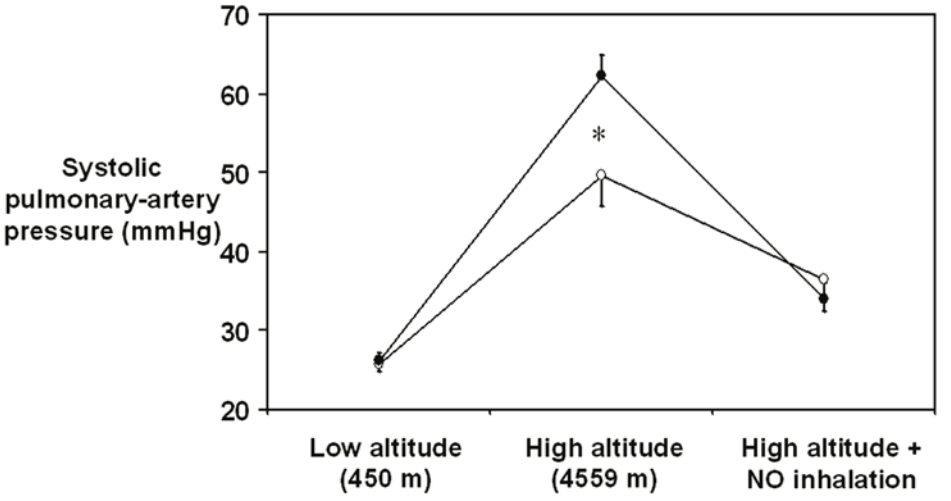

Figure 2. Effects of high-altitude exposure (4559 m) and nitric oxide inhalation at high altitude on mean[±SE] systolic pulmonary-artery pressure in 10 healthy young adults with a history of transient perinatal pulmonary hypertension (filled symbols) and in 10 control subjects (open symbols). The high-altitude induced increase in pulmonary artery pressure was significantly ($p=0.01$) larger in patients than in control subjects. During NO inhalation pulmonary artery pressure was comparable in the two groups because the NO-induced decrease in pulmonary artery pressure was much larger ($p< 0.001$) in the patients than in the control subjects (Adapted from Sartori et al. (35))

Parenthetically, exaggerated pulmonary hypertension in these young adults, while of similar magnitude as the one found in HAPE-prone mountaineers, did not cause pulmonary edema in any of these subjects.(36) This important observation suggests that exaggerated pulmonary hypertension per se may not always be sufficient to trigger HAPE, and that additional mechanisms play a role. In subsequent studies, we provided direct evidence that impaired alveolar fluid clearance (related to a defect of the transepithelial respiratory sodium transport) represents such an additional mechanism.(19, 34)

Trisomy 21

In a recent study, Durmowicz reported 6 cases of HAPE in children with trisomy 21 occurring during travel at moderate altitudes.(18) These observational studies did not establish the underlying mechanism leading to HAPE in these children. While two of these children had been treated for congenital heart defects, and thus may have suffered from a perinatal insult to the pulmonary vasculature predisposing them to exaggerated pulmonary vasoconstriction (see above), this was not the case in the other four, and alternative explanations need to be considered.

Conclusion

Based on these observations, we suggest that defective NO synthesis may represent a central event in the pathogenesis of exaggerated pulmonary hypertension during short-time hypoxic exposure. The evidence is as follows:

In HAPE-prone subjects, a condition characterized by exaggerated hypoxic pulmonary hypertension, defective pulmonary vascular endothelial and respiratory epithelial NO production impairs pulmonary vasodilation. On the other hand, loss of NO inhibition of three major pulmonary vasoconstrictor mechanisms, namely ET-1 synthesis, sympathetic central neural outflow, and oxidative stress, promotes pulmonary vasoconstriction (Figure 3).

While these important studies in HAPE-prone subjects shed light on underlying mechanisms involved in exaggerated pulmonary hypertension during short-term hypoxia, the factors or events predisposing to/protecting from exaggerated hypoxia-induced pulmonary hypertension remained largely unknown. Recent studies suggest that pathological events during the perinatal period (possibly by impairing pulmonary NO synthesis), and trisomy 21, may represent conditions predisposing to exaggerated hypoxic pulmonary hypertension.

In an attempt to translate some of this new knowledge gained during studies involving short-term hypoxic exposure to the understanding of underlying mechanisms and predisposing events of chronic hypoxic pulmonary hypertension, we have recently initiated a series of studies in high-altitude dwellers.

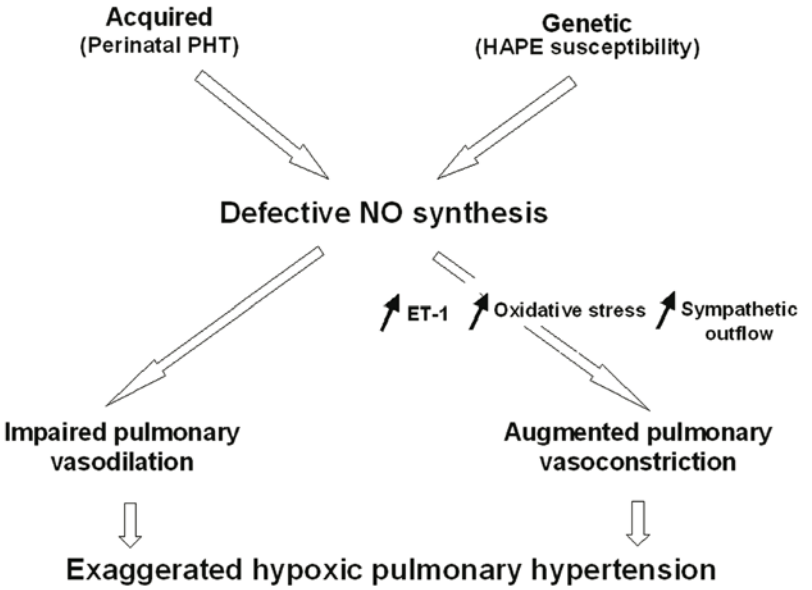

Figure 3. Mechanisms involved in the pathogenesis of exaggerated pulmonary hypertension in HAPE-prone subjects.

LONG-TERM ADAPTATION TO HYPOXIA IN HIGH-ALTITUDE DWELLERS

Due to its critical role in energy production, oxygen is essential for the survival of the cells. A reduction in tissue oxygen availability stimulates a complex series of adjustments, both at the cellular and at the systemic level. As the adaptation to hypoxia proceeds, these responses are generally limited by inhibitory feedback mechanisms. There exist, however, situations in which, for unknown reasons, these feedback mechanisms are impaired, leading to exaggerated compensatory responses to hypoxia with detrimental consequences for the organism. The underlying mechanisms regulating the delicate balance between positive (self-limited) and negative (exaggerated) adjustments to hypoxia are incompletely understood. Studies of populations permanently living at high altitude, and in particular of maladapted subgroups, may provide important insight into adaptation/maladaptation to hypoxia and, even more importantly, into underlying mechanisms involved in the pathogenesis of hypoxemia-related disease states in general.

Adaptive mechanisms differ between Tibetans, Ethiopians and Andeans

While the abovementioned studies have provided important insight regarding the cardiopulmonary adjustments to short-term hypoxia, for the clinician, the long-term adjustments to chronic hypoxia may be even more important. To this end, in collaboration with Bolivian researchers at the Instituto Boliviano de Biologia de Altura, we have recently started to study cardiopulmonary adaptation in high-altitude dwellers living in La Paz,

Bolivia (3600-4000 m). In contrast to Tibetans (normal venous hemoglobin concentration, arterial hypoxemia) and Ethiopians (normal hemoglobin concentration and normal arterial oxygenation), Andeans present the "classic" pattern of human adaptation to high altitude (erythrocytosis and arterial hypoxemia) (3, 4) making them a potentially more relevant model for hypoxemia-related disease states in lowland residents of the Western hemisphere.

Is sustained high-altitude exposure invariably associated with pulmonary hypertension?

Pulmonary artery vasoconstriction is a hallmark of the adaptation to hypoxia. It occurs very rapidly after exposure to hypoxia, and is intended to reduce blood flow through poorly ventilated alveoli. When self-limited, this vasoconstriction helps to match alveolar perfusion to ventilation. It thereby decreases the shunt effect and attenuates systemic hypoxemia. When sustained, however, hypoxic pulmonary vasoconstriction may have detrimental consequences, such as exaggerated pulmonary hypertension, right ventricular hypertrophy, and right heart failure, diseases which are associated with a high morbidity and mortality. (48)

Surprisingly little information is available on pulmonary-artery pressure in young healthy high-altitude dwellers, but the general thinking is that pulmonary hypertension is present in high-altitude native children, since invasive studies showed elevated pulmonary-artery pressure in a few children studied at high altitude in the Andes. (33, 44) However, the data to support this assumption is extremely sparse. To address this issue, we have recently measured systolic pulmonary-artery pressure in a large group of Bolivian high-altitude native children of Aymara origin in the surroundings of La Paz. Our preliminary findings indicate that pulmonary artery pressure in Aymara children is considerably lower than the one measured in Caucasian children who were born at high altitude or long-term residents of La Paz, and, indeed, appears to be quite similar to the one measured in children at low altitude. The lower pulmonary-artery pressure in the Aymara children was not related to better arterial oxygenation. Finally, there is some preliminary evidence that Aymara girls may be even better protected against hypoxic pulmonary hypertension than Aymara boys. (39) These data challenge the paradigm that sustained high-altitude exposure invariably leads to pulmonary hypertension in young healthy subjects. Protection from hypoxia-induced pulmonary hypertension may represent a specific high-altitude adaptation of the Aymaran ethnicity, but the underlying mechanism is not clear.

Does respiratory nitric oxide offset hypoxic pulmonary vasoconstriction?

In a recent study, exhaled NO at high altitude was found to be elevated in healthy Bolivian Aymara and Tibetans, when compared with a low-altitude reference sample from the United States. (5) The authors suggested that the augmented amount of NO produced in the lungs may reduce pulmonary-artery pressure in high-altitude dwellers, and improve oxygen delivery to the tissues. While this is an interesting speculation, this study does not provide any direct evidence for this concept, because measurements of pulmonary artery pressure were not performed during these observational studies. Moreover, a large part of the high-altitude dwellers (in particular among the Bolivian Aymaras) had exhaled NO concentrations that were comparable to those measured in the low altitude sample, and the

authors did not measure exhaled NO in high-altitude dwellers of Caucasian origin. Further study is needed, to determine whether augmented respiratory NO production represents a protective mechanism offsetting pulmonary hypertension in Bolivian Aymaras and/or Tibetans.

Re-entry HAPE, a marker of sustained pulmonary hypertension in high altitude dwellers?

In the surroundings of La Paz, millions of people have to deal permanently with lack of oxygen in the inspired air due to high altitude. Some high-altitude dwellers suffer from a very particular form of high-altitude pulmonary edema when they return from a sojourn at low altitude, the so called "edema pulmonar de reentrada" or re-entry HAPE. The underlying mechanism is not known. As discussed above, in mountaineers, classical HAPE is caused by the conjunction of defective alveolar fluid clearance and augmented alveolar fluid flooding related to exaggerated pulmonary hypertension. We speculated that if in high-altitude dwellers susceptible to re-entry pulmonary edema, a similar dysfunction of the pulmonary blood vessels exists, they should display a persistent elevation of the pulmonary-artery pressure at high altitude. This is exactly what we found. These subjects had markedly more elevated pulmonary-artery pressure than control subjects who never experienced re-entry pulmonary edema.(47) These important findings indicate that a history of re-entry HAPE allows to identify a subgroup of otherwise healthy high-altitude dwellers, suffering from chronic pulmonary hypertension.

The underlying mechanism causing sustained pulmonary hypertension in re-entry HAPE prone high-altitude dwellers is not known yet. Exaggerated hypoxic pulmonary vasoconstriction was not related to more severe hypoxemia, because arterial oxygen saturation in these subjects was comparable to the one observed in control subjects. Therefore, alternative explanations need to be considered. There is increasing evidence that oxidative stress may play an important role in the pathogenesis of pulmonary vasoconstriction. In fetal lambs, ductus arteriosus ligation-induced pulmonary hypertension has been found to be associated with oxidative stress, and superoxide scavengers augmented vascular relaxation to exogenous NO in this model. (9) In rats, the anti-oxidant N-acetylcysteine attenuated the hypoxia-induced pulmonary hypertension and right ventricular hypertrophy. This favourable effect appears be related to attenuation of oxidative stress, because N-acetylcysteine attenuated the hypoxia-induced stimulation of lung phosphatidylcholine hydroperoxide. (22) Finally, in humans hypoxia also appears to stimulate oxidative stress. (26) In line with this concept, preliminary data suggest that re-entry HAPE prone subjects appear to display exaggerated oxidative stress. In line with this speculation, treatment with an anti-oxidant pharmacological agent led to a dramatic decrease of pulmonary-artery pressure in a few of these subjects. Thus, these exciting preliminary findings provide the very first evidence for a pathogenic role of oxidative stress in a human form of pulmonary hypertension.(47)

The unexpected finding of a normalization of pulmonary-artery pressure in a few of these subjects suffering from chronic hypoxic pulmonary hypertension deserves an additional comment. It is generally thought that structural changes in the pulmonary vascular bed underlie chronic hypoxic pulmonary hypertension. If this were the case, pharmacological interventions would not be expected to normalize pulmonary artery pressure in these subjects. Recent studies in experimental animal models have called into question this long-held paradigm. Studies in chronically hypoxic rats, using novel techniques to exam-

ine pulmonary vascular structure, demonstrated that hypoxia did not reduce the luminal diameter. (23) In line with this concept, Rho kinase inhibitors, a vasodilating agent, rapidly normalized pulmonary artery pressure. (31) We speculate that pulmonary vasoconstriction in re-entry-HAPE-prone high altitude dwellers is functional rather than structural.

We made two additional potentially very important observations during these studies. We noticed that an unexpected large proportion of the subjects having experienced re-entry pulmonary edema in the past, were offspring of mothers suffering from preeclampsia on the one hand, and subjects suffering from trisomy 21 on the other hand.

Offspring of preeclampsia are predisposed to exaggerated hypoxic pulmonary vasoconstriction?

This intriguing observation suggested that preeclampsia may predispose the offspring to pulmonary hypertension at high altitude. In a recent field study in the surroundings of La Paz, we confirmed this finding in a larger group of children of mothers suffering from preeclampsia. The exact mechanism by which preeclampsia of the mother predisposes the offspring to hypoxic pulmonary hypertension is not clear. Our studies demonstrating exaggerated hypoxic pulmonary hypertension in young adults having suffered from transient perinatal hypoxia (see above) suggest that pathologic events during the fetal/perinatal period may leave a persistent damage to the pulmonary circulation of the newborn. It is well established that during preeclampsia, the diseased placenta produces a number of circulating molecules that are known to interfere with the vascular function of the mother. (21, 46) Most importantly, some of these molecules also have the potential to cross the placental barrier. It appears thus possible that vasculotoxic molecules produced by the diseased placenta, may cause a persistent damage to the vascular system of the fetus that under certain pathological conditions, predisposes the offspring to a pathological response later in life. Life in the oxygen poor environment of high altitude appears to represent an example of a condition that triggers such a pathological response in the pulmonary circulation.

Trisomy 21 predisposes to pulmonary hypertension in high-altitude dwellers?

Circumstantial evidence suggests that children with Down syndrome may be at risk for HAPE (see above). Exaggerated pulmonary hypertension is a prerequisite for HAPE. We, therefore, hypothesized that Down syndrome predisposes to pulmonary hypertension at high altitude. To address this issue, we recently performed echocardiographic measurements of pulmonary-artery pressure in Bolivian high-altitude natives with Down syndrome. Subjects with associated cardiac malformations were excluded from these studies. Our preliminary findings suggest that pulmonary artery pressure is considerably higher in the children with Down syndrome than in control subjects. Pulmonary hypertension in Down syndrome did not appear to be related to more severe hypoxia or increased blood viscosity, because values for arterial oxygen saturation and hemoglobin were comparable to those observed in control subjects. These findings suggest that Down syndrome predisposes to pulmonary hypertension at high altitude. The underlying mechanism is not clear yet. We speculate that smaller than normal lungs and cross-sectional area of the pulmonary vascular bed and/or endothelial dysfunction related to augmented oxidative stress, which are both associated with trisomy 21, could be factors predisposing to exaggerated hypoxia-

induced pulmonary hypertension in Down syndrome.

With regard to the latter, there is evidence that augmented oxidative stress, is involved in the pathogenesis of the central neural and ocular manifestations of trisomy 21. (8, 12, 24) Augmented oxidative stress in trisomy 21(27) has been attributed to augmented generation of hydrogen peroxide and hydroxy radicals related to the extra gene for superoxide dismutases located on the chromosome 21. (10) We speculate that in subjects with trisomy 21, augmented oxidative stress may also predispose to pulmonary hypertension in high -altitude dwellers. In line with this speculation, the antioxidant vitamin C decreased pulmonary artery pressure in children with trisomy 21 living at high altitude, whereas it had no detectable effect on pulmonary artery pressure in control subjects.

Pulmonary hypertension associated with erythrocytosis (Monge disease)

Erythrocytosis is another well known adaptive mechanism to chronic hypoxia. As for hypoxia-induced pulmonary vasoconstriction, the beneficial effects of this compensatory adjustment are lost, if this adjustment is non-limited, as evidenced by the Monge disease. The large majority of these subjects also suffer from severe pulmonary hypertension and right ventricular failure. The underlying mechanisms are unclear. It appears possible that reduced NO bioavailability, related to erythrocytosis, may contribute to pulmonary hypertension in Monge disease, since the rapid oxidation of nitric oxide by oxyhemoglobin to form methemoglobin and nitrate, could limit the magnitude and duration of NO-induced vasorelaxation. (13, 14) Moreover, the higher viscosity per se may contribute to augmented vascular resistance in these patients.

CONCLUSION

Based on our results, we suggest the following new concepts regarding the regulation of pulmonary-artery pressure in high-altitude dwellers (Figure 4).

In contrast to the long-held belief, pulmonary-artery pressure does not appear to be elevated in young healthy children of Aymara origin living between 3’600 and 4’000 meters. The situation appears to be different for subjects of Caucasian origin living at high altitude who display pulmonary hypertension. The mechanism protecting Aymaras from hypoxic pulmonary hypertension is not clear, but possibly could include augmented pulmonary NO synthesis.

Preliminary studies have identified, for the first time, 3 subgroups of high altitude dwellers suffering from sustained pulmonary hypertension, namely, re-entry-HAPE-prone subjects, offspring of preeclampsia, and subjects with trisomy 21. The underlying predisposing mechanisms are not known yet, but augmented oxidative stress (facilitated by impaired pulmonary NO synthesis?) may represent a candidate mechanism.

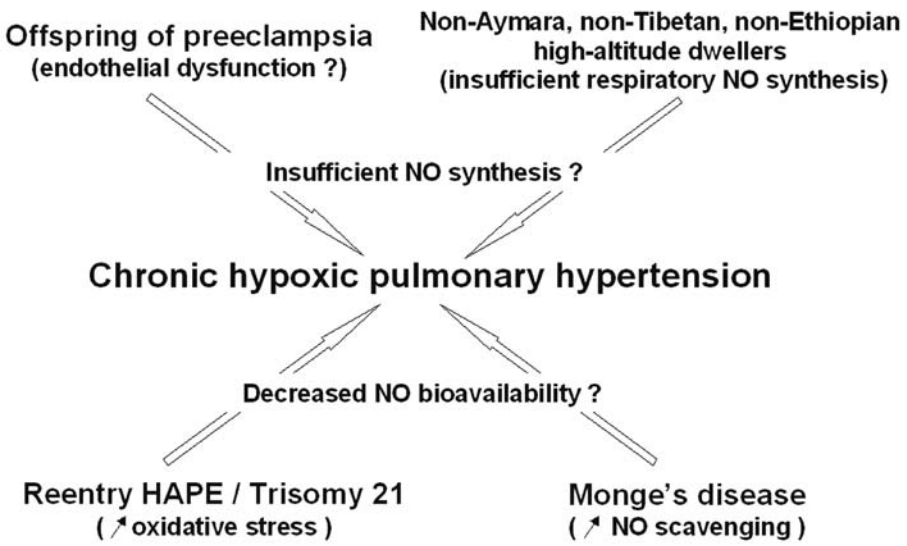

Figure 4. Mechanisms/factors predisposing to sustained pulmonary hypertension in high-altitude dwellers.

ACKNOWLEDGMENTS

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Chapter 24

GENE HUNTING IN HYPOXIA AND EXERCISE

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Abstract: New technologies in genomics and proteomics are revolutionizing the study of adaptation to environmental stress. These approaches provide a comprehensive overview of the responses of thousands of genes/proteins to stress and enormously expand our view of the molecular and metabolic changes that underlie physiological responses. Several new technologies can help physiological labs to become gene hunters. DNA array screening is particularly effective for two purposes: (1) identifying coordinated responses by functional groups of gene/proteins such as multiple members of a signal transduction cascade or enzymes of a metabolic pathway, and (2) highlighting cell functions that have never before been linked with the stress under consideration. We have shown that heterologous screening of DNA arrays can be a highly effective method of gene hunting for the comparative biochemist provided that it is followed up by species-specific analyses including PCR to quantify transcript levels and Western blotting to analyze protein responses. Recent work in my lab has used cDNA array screening to evaluate responses to low oxygen by multiple hypoxia/anoxia tolerant systems, revealing common gene responses across phylogeny. Analysis of vertebrate facultative anaerobiosis in freshwater turtles reveals an interesting mixture of gene responses, including up-regulation of antioxidant enzymes, protease inhibitors, and proteins of iron metabolism; a few of these are coordinated by the hypoxia inducible factor in other systems but most are not. Array screening is also providing new insights into how exercise stimulates the growth of differentiated muscle cells and studies in our lab are identifying the gene responses associated with "anti-exercise" – gene up-regulation that aids hibernating mammals to maintain their muscle mass despite months of inactivity.

Key Words. anoxia tolerance, hibernation, cDNA array screening, metabolic rate depression

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INTRODUCTION

Major advances in molecular biology technology over the last decade have fundamentally changed the approach that my lab uses to identify biochemical adaptations that support animal survival in extreme environments. We have become gene hunters. The traditional approach to studying stress responses by cells and organisms has been a "top-down" one – begin with a highly "visible" phenotypic response (e.g. 2 M glycerol accumulated by a cold-hardy insect, a massive synthesis of heat shock proteins, etc.) and work backwards to trace the protein/enzyme and gene modifications that underlie it. This approach continues to be excellent in many cases but has one key limitation in that to begin it requires the identification of a major metabolic response (phenotype change) in either qualitative or quantitative terms. Sometimes this leads to a perhaps inordinate focus on a selected phenotypic response (e.g. thousands of papers on cryoprotectant levels in cold-hardy animals) that can obscure or delay discovery of other adaptive responses that are just as important to stress survival but have no readily detectable "signature". Gene hunters take a different approach. Using multiple new technologies, particularly DNA microarray screening, organismal response to a stress can be examined in a "bottom-up" direction that provides an unbiased assessment of the responses to stress of thousands of different genes, representing hundreds of different cell functions, and reveals the totality of gene expression changes that underlie the physiological response to stress.

The two most important gene discovery techniques available to the gene hunter are cDNA library screening and DNA microarray screening and we have used both in our analysis of diverse animal models of anoxia tolerance, freeze tolerance, cold hardiness and hibernation (7,44-47,49). Construction and screening of a cDNA library is an arduous job and screening favours the discovery of high abundance mRNA transcripts but the technique has one key advantage for researchers working with unusual animal models. That is the opportunity to find novel genes that are unique to the species/stress under study. For example, our screening of a cDNA library made from tissues of the anoxia tolerant marine snail, *Littorina littorea*, identified two anoxia-responsive genes that have, to date, no equivalents in other species (28,30,45). We have not yet defined their functions but structural features of the gene *kvn* suggest that it may encode an iron-sulfur protein related to the ferredoxin family (28) whereas *sarp* contains two putative EF-hand calcium binding domains that suggest a role for SARP-19 protein in calcium-activated signalling or calcium sequestering during anaerobiosis (30). Similarly, our cDNA library screening studies of freeze tolerant wood frogs discovered three novel freeze-responsive genes of as yet unknown functions but that encode proteins with distinctly different physical characteristics, organ distribution, and responses to intracellular second messengers (47).

DNA microarray screening provides even greater opportunities for the gene hunter. The number of species for which homologous arrays are available is growing rapidly and provides the opportunity to screen the responses to an imposed stress by thousands of genes. Major effort is being put into perfecting array screening in both qualitative and quantitative terms in order to be able to rely on the technology as a predictive and diagnostic tool, particularly in medicine. We have taken a different approach and shown that excellent information can also be derived from heterologous screening using arrays made from cDNA of one species (e.g. human, rat, *Drosophila*) but probed with cDNA of another (7,47,49). Clearly, because of DNA sequence differences, cross-hybridization during heterologous

probing is never 100%. A substantial number of genes on the array will go unevaluated (including some that may be stress-responsive) and a potential for false positives also exists (experimental cDNA binding to a sequence that is not its gene homologue). However, we have found that good levels of cross-hybridization can be achieved during heterologous probing, particularly after initial manipulations of hybridization and washing conditions to optimize binding. For example, we used human 19,000 gene chips from the Microarray Centre (Ontario Cancer Institute, Toronto; *http://www.microarrays.ca/*) to screen for anoxia-, freeze-, or hibernation-responsive gene expression in multiple systems, with cross-hybridization percentages of 85-90% using cDNA from hibernating mammals (ground squirrels or bats), 60-80% for frog tissues, and 18.35% for hepatopancreas of the marine snail *Littorina littorea*) (7,45-47). Although this last number for snails seems low, we were still able to evaluate the expression of nearly 3500 genes on the 19K array and found over 300 that were putatively up-regulated by anoxia exposure, providing many new leads for follow-up in the hunt for genes that support anoxia survival in molluscs.

Heterologous screening is not appropriate for gathering quantitative data – that is derived from various follow-up techniques (see below) – but this form of screening is excellent in qualitative terms. Gene hunting by this method is particularly exciting for two reasons: (a) the hunt often reveals previously unsuspected genes and proteins (and their corresponding cell functions) that participate in the adaptive response, and (b) the responses of related groups of genes (proteins) can be assessed – e.g. families of proteins, components of signal transduction cascades, enzymes of a metabolic pathway – to gain a broad overview of the areas of cell function that respond in creating the coordinated adaptive response to stress. These features are key to allowing us to build a "big picture" of gene responses to an imposed stress and they point the way into targeted studies of specific areas of metabolism.

For example, our recent analyses of hibernating ground squirrels and anoxia tolerant turtles (44,46) showed the completely unexpected up-regulation in both systems of selected *ser*ine *p*rotease *in*hibitor*s* (serpins) in the experimental state (torpid or anoxic), suggesting that these have a role to play in animals entering hypometabolism (a key survival strategy of both hibernation and anaerobiosis is metabolic rate suppression (51)). Most serpins are plasma proteins (typically secreted by liver) that act as irreversible covalent inhibitors of proteases that cleave specific proteins. Many inhibit the proteases that act at critical checkpoints in self-perpetuating proteolytic cascades such as the proteases involved in blood coagulation, fibrinolysis, inflammation, and complement activation (16). Enhanced levels of selected serpins during entry into a hypometabolic state could be key for inhibiting specific proteolytic reactions and cascades that could otherwise cause damage to tissues during long term hypometabolism. Suppression of proteolytic cascades as part of metabolic rate depression would also contribute to lowering net protein turnover during hypometabolism, thereby contributing to ATP savings (44,51).

Because of the nature of heterologous array screening, the information that derived from arrays must be confirmed, validated and quantified by other means. Typically genes showing at least 2-fold higher signal in experimental versus control states are good candidates for follow-up studies. The basic plan that we have used for follow-up in recent studies (9,10,34) includes the following: (1) design of DNA primers for the gene of interest based on a consensus sequence put together from available sequences in Genbank, (2) use of the primers to retrieve the species-specific cDNA via PCR, (3) nucleotide sequencing of the retrieved cDNA to confirm its identity followed by design and synthesis of a spe-

cies-specific probe, (4) use of the species-specific probe to evaluate organ-, time-, and stress-specific gene expression via Q-PCR or RT-PCR, (5) as appropriate, 3' or 5'RACE to retrieve the full nucleotide sequence for analysis of possible adaptive changes in amino acid sequence compared with other species, and (5) use of the putative amino acid sequence to design and synthesize peptide antibodies (or use of commercial antibodies if appropriate) to quantify patterns of protein expression. This latter is especially important because, while it is generally true that enhanced gene expression leads to elevated protein levels, exceptions occur and so quantification of relative mRNA levels in control versus stress situations does not always correlate with changes in protein levels. Indeed, some proteins are subject to primary regulation at other levels, through regulation of translation (e.g. ferritin) or proteoloysis (e.g. hypoxia-inducible factor 1α [HIF-1α]) (17,18). Our studies with hypometabolic systems have also noted instances of time-frame differences between transcription and translation. For example, in kidney of hibernating ground squirrels, mRNA transcripts of the organic cation transporter type 2 (*Oct2*) were 2-3 fold higher than in euthermic animals as assessed by both Northern blots and cDNA arrays but the transcripts remained sequestered with translationally silent monosomes during torpor (23). As a result, Western blots showed that OCT2 protein levels in kidney dropped by 66% during hibernation but after arousal, newly assembled polysomes can quickly produce OCT2 protein from existing *Oct2* transcripts.

A few final points about array screening can be made. The technology for array screening is now widespread and highly automated and screening is offered as a service by many companies. My lab now finds it cost-effective to out-source our screening needs rather than invest in expensive equipment and expensive mistakes while training personnel. We use array screening as the initial identification tool to highlight interesting genes but then turn to other techniques to validate array results and to further investigate the regulation and the function of the interesting genes/proteins. Hence, the modern lab in molecular physiology need only invest in validation technologies such as PCR for quantifying stress-induced changes in mRNA transcript levels and Western blotting for analyzing the corresponding changes in protein levels. These technologies, together with activity assays and other investigative techniques, are the ones that are needed to analyze the functional role of gene/protein expression

Other new technologies are also available to gene hunters to gain information about the regulation of the gene/protein of interest. A cDNA sequence can be used as the starting point to screen a genomic library to retrieve the full genomic sequence (introns plus exons) as well as promotor (5'untranslated) regions. Genomic sequence is often crucial to understanding the evolution of novel genes (13) and identification of transcription factor binding sites in the promoter region unlock regulatory aspects of gene function. Bioinformatics programs can be applied to analyze amino acid sequences to detect regulatory motifs (e.g. phosphorylation sites) and to analyze species-specific differences in amino acid sequence that may be adaptive (21,30). Rapidly advancing proteomics technologies are also making it increasingly easy to directly analyze stress-responsive changes in the proteome (Figure 1)(10,32). Proteins are separated by two dimensional gel electrophoresis and protein spots that differ in intensity between control and experimental states are excised, digested, and analyzed by mass spectrometry based methods such as MALDI-TOF for peptide mass fingerprinting and tandem MS/MS for de novo peptide sequencing (32). Peptide fingerprints are now known for hundreds of cellular proteins so that identification can often be achieved

by evaluating the pattern alone or with the additional selective sequencing of one or more of the peptide peaks. Proteins that are subject to reversible phosphorylation can also be studied using phospho-specific antibodies (raised against the peptide segment containing the phosphorylated amino acid) to quantify stress-responsive changes in the relative level of phosphorylated versus dephosphorylated protein which typically mirrors the activity state of the protein (Figure 1). Use of phospho-specific antibodies is especially key for tracing multi-component signal transduction cascades leading from cell surface to nuclear gene activation. Hence, we now have the molecular tools to evaluate almost any metabolic system and to search for the breadth and depth of biochemical adaptations that define the differences between stress-tolerant and stress-intolerant organisms.

GENE HUNTING IN HYPOXIA AND ANOXIA

The strategies and approaches for gene hunting outlined above are being used to explore animal responses to low oxygen and illuminate differences between hypoxia/anoxia tolerant and intolerant species. Most organisms are adversely affected by low oxygen availability because the supply of ATP from mitochondrial oxidative catabolism is reduced and falls below the pace of cellular ATP consumption; if not corrected, this imbalance can become lethal rapidly. The response to low oxygen by most vertebrates, including man, is a suite of compensatory strategies at behavioural, physiological and biochemical levels that address two main goals: (1) increase oxygen delivery to tissues, and (2) elevate glycolytic ATP output. These strategies are accomplished in part by increased transcription and translation of genes under the control of the HIF-1 transcription factor (5,17,54). The HIF-1α subunit is oxygen sensitive. At high oxygen tensions, O_2-dependent hydroxylation of two proline residues sets up the alpha subunit for rapid proteolysis so that net HIF-1α levels in cells remain low whereas under low oxygen conditions HIF-1α is stabilized, dimerizes with HIF-1ß, and activates gene transcription. The list of HIF-1 regulated genes is large and includes most glycolytic enzymes as well as glucose transporter isoforms 1 and 3. Various proteins associated erythropoiesis and capillary growth are also up-regulated under direct control by HIF-1α or secondary control by erythropoietin or vascular endothelial growth factor (VEGF), themselves both regulated by HIF-1 (5,17,54).

Compensatory strategies allow many species to adjust to short or long term reductions in oxygen availability (hypoxia) and even brief periods of anoxia but some animals are challenged by environments or lifestyles where oxygen deprivation is continuous for days, weeks or months. Survival of long term oxygen lack requires a different strategy – a conservation strategy that dramatically lowers tissue energy needs to a level that can be sustained over the long term by anaerobic pathways of ATP production. Research over the last 20-30 years by my lab and others has illuminated many of the biochemical adaptations and regulatory mechanisms that support facultative anaerobiosis (for review 2,25,31) and emphasized the importance of metabolic rate depression (often by >90%) as the key element for energy savings in anoxic state (51).

The premier facultative anaerobes among vertebrate species are various freshwater turtles of the *Trachemys* and *Chrysemys* genera (25,26). Adults can typically survive in cold deoxygenated water (<10°C) for ~3 months. They do so by lowering their metabolic rate to ~10% of the corresponding value that they exhibit in air at the same temperature.

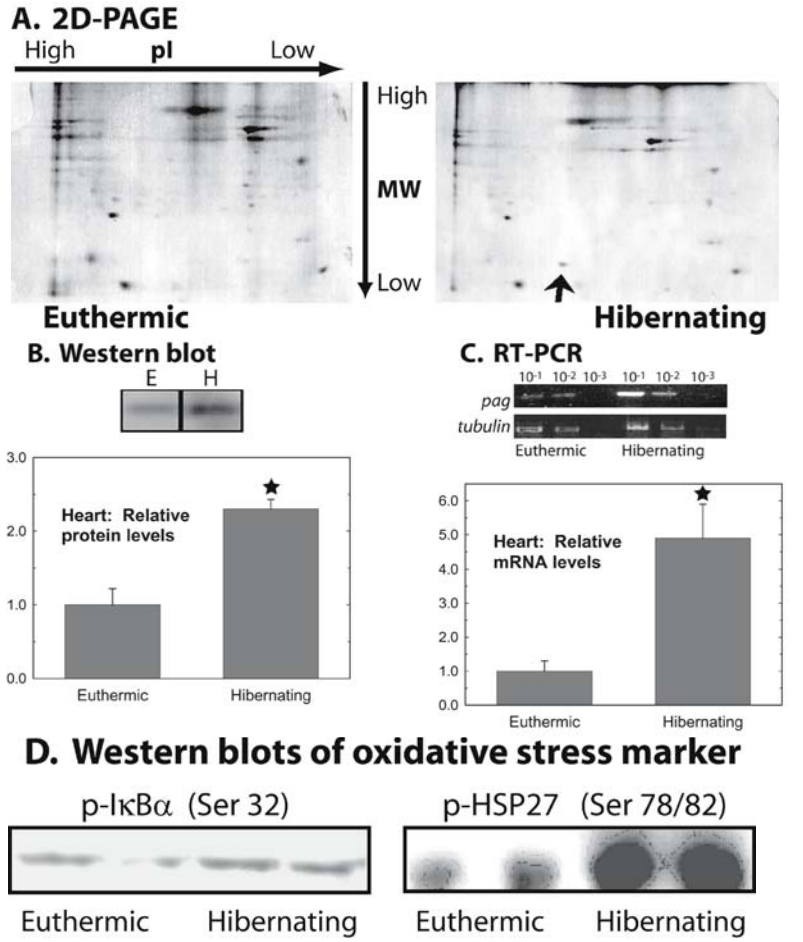

Figure 1. Strategies for stress-responsive protein hunting: identification and analysis of a protein called proliferation-associated gene (PAG) (also known as thioredoxin peroxidase 1 or peroxiredoxin 1) in heart of hibernating bats, *Myotis lucifugus*. (A) Two dimensional polyacrylamide gel electrophoresis separated 150 μg aliquots of heart soluble protein. Coomassie blue staining revealed stronger expression in hibernator heart of a protein with an isoelectric point of ~7 and a molecular weight of ~22 kD (indicated by arrow). The protein spot was excised, subjected to tryptic digestion, analyzed by mass spectrometry and two tryptic peptides were obtained (GLFIIDGK and QITVNDLPVGR) and used for identification. (B) Western blotting with antibody to human PAG showed 2.30 ± 0.13 fold (n=3, $P<0.05$) higher protein in heart of hibernating versus euthermic bats. (C) Consensus primers for the *pag* gene were designed from the sequences of human, rat and mouse *pag* and used to clone bat *pag*. The full nucleotide sequence was obtained (Genbank accession number AY680839), showing 92% nucleotide and 99% amino acid sequence identity with the human protein. RT-PCR was performed on three dilutions of bat *pag*; amplification of α-tubulin message in the same samples was used to normalize *pag* expression levels. Using data from the 10^{-2} dilution, bat *pag* transcripts were 4.9 ± 1.6 fold (n=3, $P<0.05$) higher in heart of hibernating animals. (D) Protein markers of oxidative stress in bat heart were assessed by Western blotting. Antibodies assessed relative amounts of phosphorylated IκB-α(Ser32) and phosphorylated HSP27(Ser78/82) in euthermic and hibernating heart. Complied from (10).

Huge organ reserves of glycogen are mobilized to fuel glycolytic ATP production and metabolic poisoning from lactate build-up is avoided by two measures: (a) buffering by calcium and magnesium carbonates released by the shell, and (b) storing a high percentage of the lactate produced in the shell (26). Metabolic rate depression is achieved by targeting and coordinating the rates of energy-producing pathways and energy-utilizing cell functions to "turn down the fires of life" and reorganize the priorities for ATP expenditure (2,51). Studies with isolated turtle hepatocytes provide an excellent example of this. Incubation of these cells under anoxic conditions decreased ATP turnover by 94% and dramatically changed the portion of ATP turnover that was devoted to five main ATP-consuming processes: ion motive ATPases, protein synthesis, protein degradation, gluconeogenesis, and urea synthesis (24). As a result, the Na^+K^+ATPase pump became the dominant energy sink in anoxic hepatocytes, consuming 62% of total ATP turnover compared with 28% in normoxia. Protein synthesis and degradation were largely shut down (by >90%) and urea synthesis was halted. A primary mechanism used for metabolic suppression is reversible protein phosphorylation, using the addition (by protein kinases) or removal (by protein phosphatases) of covalently-bound phosphate to partially or fully inactivate proteins/enzymes. This mechanism reappears across phylogeny as the means of making major changes in metabolic rate in numerous animal models of metabolic suppression including hibernation, estivation, diapause, dormancy, torpor, anhydrobiosis and anaerobiosis (51). Reversible phosphorylation controls have been linked with the suppression of multiple cell functions during anoxia in turtles including glycolytic enzymes, voltage-gated ion channels (Na^+, Ca^{2+}, K^+), membrane receptors (e.g. *N*-methyl-D-aspartate-type glutamate receptor), and protein synthesis (e.g. ribosomal initiation and elongation factors) (2,25,51). Another consumer of cellular energy is gene transcription and, not surprisingly, the global rate of gene transcription is suppressed in hypometabolic states. This is done with little net change in total mRNA which is sequestered intact in association with translationally-silent monosomes or ribonuclear proteins and preserved until polysomes can be reassembled again (51). However, selected genes are also specifically repressed or activated to resculpt organs for long term anaerobic survival. For example, in anoxic turtle brain, mRNA transcripts of voltage-dependent potassium channels were reduced to just 18.5% of normoxic levels after 4 h of anoxia but rebounded after reoxygenation (37).

Of greater interest to us are the genes that are up-regulated under anoxia. Clearly, these must important to the anaerobic phenotype because gene transcription and protein translation are ATP-expensive processes that are minimized under anoxia (24). Hence, ATP would not be wasted on the synthesis of nonessential proteins. Furthermore, we would not expect anoxia-responsive genes in facultative anaerobes to be those that are up-regulated under HIF-1 control in hypoxia-sensitive species. Anoxia tolerant species do not increase their net glycolytic ATP production; indeed, they reduce it during anaerobiosis (51). Furthermore, both erythropoiesis and capillary growth during natural hypoxia/anoxia excursions is counter-intuitive as it is energy-expensive at a time when energy savings are crucial and unproductive since low tissue oxygen levels cannot be improved by enhanced oxygen delivery under apnoic, anoxic conditions.

So, what genes do turtles, the champion vertebrate facultative anaerobes, up-regulate when challenged with low oxygen stress? Quite a variety as it turns out. Our studies using cDNA library and DNA array screening tell an interesting story about which cell functions need adjustment for anoxia survival. Our first work on anoxia-induced gene expression

focused on submergence anoxia (20 h in N_2-bubbled water at 7°C) in adult red-eared sliders, *Trachemys scripta elegans*. Differential screening of a cDNA library made from heart produced a surprising result – up-regulation of two genes encoded on the mitochondrial genome: *Cox1* that encodes cytochrome C oxidase subunit 1 (COX1) and *Nad5* that encodes subunit 5 of NADH-ubiquinone oxidoreductase (ND5) (4). Transcripts rose within 1 h of anoxia exposure to 3-4.5 fold higher than aerobic control values, remained high after 20 h, and then declined during aerobic recovery. Expression of both genes also rose during anoxia in muscle, brain and kidney. A second study found that other mitochondrially encoded genes were anoxia responsive in liver: transcripts of *Cytb*, encoding cytochrome b, and *Nad4*, encoding subunit 4 of ND, rose by 5- and 13-fold, respectively, within 1 h under anoxia (55). The reason for mitochondrial gene up-regulation in anoxia is not yet known but the phenomenon also occurs during freezing (which causes ischemia) in freeze tolerant turtles and wood frogs (4,47) and in mammalian hibernation (43,51).

In recent work we have applied cDNA array screening to an evaluation of anoxia-responsive gene expression in both adult *T. s. elegans* and hatchling painted turtles, *Chrysemys picta marginata*. The young of this species are remarkable because, after hatching in September, they remain in their shallow nests (<10 cm deep, typically on exposed river or lake banks) over their first winter and employ strategies of cold hardiness to survive at temperatures below 0°C. Near the northern limits of their range, such as in Algonquin Park, Ontario where we study them, the hatchlings survive freezing, enduring the conversion of ~50% of total body water into extracellular ice (50). A significant element in natural freeze tolerance is ischemia/anoxia resistance necessitated because plasma freezing halts oxygen delivery to organs. We used heterologous screening with human 19K gene chips to search for differential gene expression in adult *T. s. elegans* given 4 hours of anoxic submergence in nitrogen-bubbled water and hatchling *C. p. marginata* given 4 hours exposure under a nitrogen gas atmosphere, each compared with aerobic controls and all animals held at 7°C.

ANOXIA RESPONSIVE GENE UP-REGULATION IN TURTLE HEART AND LIVER

Array screening of heart and liver of hatchling *C. p. marginata* revealed a variety of genes that showed enhanced expression in anoxic turtles, binding intensities of labeled probe being 1.5-3.5 fold higher than in aerobic controls. Of these, I will comment here on three outstanding groups of genes that were consistently up-regulated: (1) iron storage proteins, (2) enzymes of antioxidant defense, and (3) selected serpins.

Iron storage proteins: Hatchling turtle heart and liver both showed enhanced expression of ferritin heavy (H) and light (L) chains and transferrin receptor 2 (TfR2) during anoxia exposure. Iron is a vital component of many proteins, including cytochromes and haemoglobin, but free iron in the ferrous state (Fe^{2+}) participates in the Fenton reaction with hydrogen peroxide and lipid peroxides to generate highly reactive hydroxyl radicals and lipid radicals (18,20). Hence, intracellular free iron levels are kept low by storing the metal in ferritin, a huge protein consisting of 24 H and L subunits that surrounds a core of up to 4500 iron atoms locked in a low reactivity ferrihydrite state (18). In plasma, iron is bound tightly to another protein, transferrin (Tf), and iron uptake into cells involves endocytosis

of Tf after docking with the cell surface transferrin receptor (TfR). Acidification of the endosomes releases the iron and Tf and TfR are recycled. The TfR1 isoform is ubiquitous but in mammals TfR2 is restricted to tissues such as liver and erythroid cells. Both ferritin and TfR proteins are induced by hypoxia in mammals and the Tf and TfR genes are under HIF-1 control (41,52,54). What is the reason for ferritin and TfR2 up-regulation under anoxia in turtles? Clearly, an increase in cellular iron uptake and storage is indicated. Interestingly, in comparable studies of anoxia tolerance in marine snails, we also found anoxia-responsive up-regulation of ferritin H chain mRNA and protein and ferritin mRNA remained in the polysome fraction during anoxia, indicating that it is one of just a few actively translated proteins in anoxia (29). It is doubtful that the purpose of ferritin up-regulation in anoxia tolerant species is related to haemoglobin production; one would expect biosynthesis to strongly suppressed in anoxic turtles and snails do not have haemoglobin. Alternative reasons for iron sequestering in anoxia tolerant animals could include: (a) to minimize free Fe^{2+} so as to limit the potential for generating reactive oxygen species (ROS), particularly when oxygen is rapidly reintroduced into tissues, or (b) storage of excess iron during hypometabolism when the net rate of biosynthesis of iron-containing proteins is low. The first reason seems quite likely especially when put together with the simultaneous up-regulation of antioxidant genes in turtle organs.

Antioxidant defenses: Anoxia exposure stimulated up-regulation of several antioxidant enzymes (AOEs) in heart and liver of hatchling *C. p. marginata*: superoxide dismutase 1 (SOD-1), glutathione peroxidase (GPX) isozymes 1 and 4, glutathione-S-transferase (GST) isozymes M5 and A2, and peroxiredoxin 1. Many of the damaging effects of hypoxia or ischemia in mammals can be traced to ROS attack on cellular macromolecules occurring particularly during the transition from hypoxia/ischemia back to normal oxygen levels. This is because cellular antioxidant defenses can be overwhelmed by a burst of ROS generation when oxygen levels rise rapidly (20). Hence, hypoxia/anoxia survival requires not just adaptations that deal with problems of the anoxic state (low ATP availability, toxicity of accumulated end products, etc.) but also adaptations that deal with the reintroduction of oxygen (19,20). Studies by our lab and others have shown that anoxia tolerant species have two strategies for dealing with oxidative stress during natural transitions from low to high oxygen availability: (a) high constitutive antioxidant defenses, both AOEs and metabolites, and (b) inducible increases in AOE activities typically occurring in the low oxygen state in anticipation of their need when oxygen levels rise again (19,20). Indeed, the best facultative anaerobes that experience routine anoxic excursions, such as adult *T. s. elegans*, have constitutive AOE activities that are comparable to values seen in mammals and many-fold higher than in other cold-blooded vertebrates (20). Species that face anoxia less often show anoxia-induced enhancement of defenses. The responses of the hatchling *C. p. marginata* indicate readily inducible antioxidant defenses, a response that would be important for enduring ischemia/reperfusion insults during winter freeze/thaw exposures. The very high constitutive antioxidant enzyme activities characteristic of adult turtles would probably appear only in subsequent years when the animals are challenged by routine low oxygen excursions during diving or wintering underwater.

The up-regulation of peroxiredoxin 1 deserves a special mention because it may indicate a new principle of metabolic adjustment for anoxia tolerance. The traditional view of ROS is that they are bad and the purpose of antioxidant defenses, therefore, is to prevent

or repair the damage done by ROS to cellular macromolecules. This view began to change when it was recognized that nitric oxide ($^{\bullet}NO$) and peroxynitrite ($OONO^{-}$) have second messenger functions and is now undergoing radical revision with the recent demonstration that both superoxide and hydrogen peroxide are effective intracellular second messengers (39). Therefore, it would be expected that one or more enzymes would be present in cells to modulate the levels of these ROS with respect to their signalling functions. Peroxiredoxins (Prx) may have this role (in addition to a general role in antioxidant defense). They are relatively recent additions to the known antioxidant defenses of cells (15,57). Six isoforms of Prx occur in mammals, all containing a reactive Cys in a conserved region near the N-terminus that can reduce H_2O_2 or various alkyl hydroperoxides. Prx1, which is anoxia-responsive in both heart and liver of turtles, is a cytosolic dimer that uses either thioredoxin or reduced glutathione (GSH) as the electron donor. Prx1 is induced by oxidative and other stresses on cells and cells transfected with Prx1 exhibit resistance to apoptosis caused by hydrogen peroxide (15). Substantial support for H_2O_2 as an intracellular second messenger has accumulated recently including: (a) transient elevations of intracellular H_2O_2 occur in response to various cytokines and peptide growth factors, (b) elevated H_2O_2 affects the function of various protein kinases and phosphatases, transcription factors, and G proteins, and (c) inhibition of H_2O_2 generation results in a complete blockage of signalling by growth factors including PDGF, EGF and angiotensin II (39). The overriding critical mechanism of anoxia tolerance is metabolic rate depression – the coordinated suppression of the rates of ATP-producing and ATP-utilizing to stabilize a new much lower rate of ATP turnover (51). As noted earlier, biosynthetic processes (e.g. protein synthesis) are major targets of suppression when ATP is limiting (24,51). Predictably, then, another element of metabolic suppression should be a blockage of cell responsiveness to growth signals. One way to do this could be to strongly suppress the levels of intracellular second messengers, such as H_2O_2, that mediate growth factor and cytokine effects. Up-regulation of Prx1 could facilitate this as Prx1 is known to catabolize H_2O_2 produced from cell surface signalling (39). Prx1 is also regulated by cyclin dependent kinases; Cdc2 kinase-mediated phosphorylation reduced Prx1 activity by 80% and Prx1 was phosphorylated in cells in mitotic phase but not during interphase.

Serpins: As mentioned earlier, serpins are specific inhibitors of selected proteolytic enzymes, often of proteases circulating in the plasma. Most are synthesized and secreted by liver. Anoxia exposure of adult *T. s. elegans* turtles triggered enhanced expression of selected serpins in liver and heart: SERPINC1 (antithrombin), D1 (heparin cofactor II; liver only), and F1 (pigment epithelium derived factor (PEDF)) as well as another inhibitor, called tissue factor pathway inhibitor (46). Our analysis of hatchling *C. p. marginata* also found enhanced expression of SERPINC1 and D1 expression in liver and SERPINF1 and G1 (complement inhibitor) in heart of anoxia exposed turtles, compared with aerobic controls. SERPINC1 and SERPIND1 both inhibit thrombin and, thereby, suppress the clotting cascade. Predictably, clotting capacity should be reduced during anaerobiosis to minimize the risk of thrombosis in the microvasculature under the low blood flow conditions caused by bradycardia during hypometabolism.

SERPINF1 or PEDF is also interesting. It does not inhibit a protease but instead antagonizes VEGF with potent anti-angiogenic effects that have been shown to inhibit vascular growth in several tissues (6,35). HIF-1 triggers a wide range of gene responses, not all of

which may be needed in any given situation of low oxygen availability. Anoxia tolerant organisms must clearly retain and use HIF-1 in many circumstances (e.g. to regulate vascular growth during development or erythropoiesis after blood loss) but under environmental anoxia, up-regulation of some of the genes under HIF-1 appears appropriate (e.g. TfR) but others seem counter-intuitive (e.g. VEGF to trigger vascular growth). The putative up-regulation of PEDF in both adult and hatchling anoxia-tolerant turtles is highly intriguing for it suggests a way of blocking an unnecessary angiogenic response during natural anaerobic excursions.

GENE HUNTING IN EXERCISE AND "ANTI-EXERCISE"

The principles and methods for gene hunting are applicable to analyzing the effects of all forms of physiological and environmental stress on organisms. Muscle metabolism is no exception. Many recent studies have applied microarray screening to analyzing multiple aspects of human muscle performance (38) such as the patterns of gene changes seen during endurance exercise (14,56). For example, in a comparison of endurance trained versus untrained cyclists, trained athletes showed significantly higher levels of mRNA for eight genes including DNA repair enzymes, transcription factors, signal transducers, and a glycolytic enzyme (56). Endurance exercise increases the volume density of type I (red) fibers and their mitochondrial volume density and capillarity. Capacity for the oxidation of both fatty acids (lipoprotein lipase, acyl-CoA dehydrogenase, carnitine palmitoyl transferase, fatty acid translocase) and glucose (GLUT4 transporters, hexokinase) increases and Krebs cycle (pyruvate and succinate dehydrogenases) and electron transport enzymes (e.g. COX1, COX4, ND6) are elevated (14).

Studies in my lab have been examining the gene responses of what could be the ultimate "couch potato" or anti-exerciser – the hibernating mammal. Species such as ground squirrels and bats can spend 6-9 months of the year hibernating; long bouts of cold torpor (days-weeks) are interspersed with brief periods of arousal (a day or less) back to euthermic conditions. During torpor, metabolic rate drops to 1-5% of normal, physiological functions (e.g. heart beat, breathing, kidney filtration, and others) are strongly suppressed, body temperature (Tb) can drop to near 0°C, and skeletal muscles go largely unused (53). Hibernators experience some muscle atrophy, but it is much less than would expected from equivalent periods of inactivity in humans (11,42). Gene hunting techniques can provide the answers to multiple questions about skeletal muscle responses during hibernation including: (a) how is torpor induced and coordinated among all cell functions, (b) how are the normally injurious effects of hypothermia on mammalian tissues avoided, (c) how are fuel metabolism and cellular energetics managed, and (d) how is atrophy minimized.

Gene expression supporting hibernation can occur on two time scales: (a) seasonal, and (b) induced during entry into each torpor bout. Recent studies by my lab and others have used diverse techniques of gene screening to analyze the latter in ground squirrels. To date, known hibernation responsive genes have been found in many organs including α_2-macroglobulin in liver, moesin in intestine, isozyme 4 of pyruvate dehydrogenase kinase (PDK4) and pancreatic lipase in heart, isoforms of uncoupling proteins (UCPs) and fatty acid binding proteins (FABPs) in multiple tissues, the ventricular isoform of myosin light chain 1 ($MLC1_v$) in heart and skeletal muscle, six kinds of membrane transporters in kidney, the

melatonin receptor, eight types of serpins in multiple organs (A1, A3, A7, B9, C1, E2, F2, G1), several antioxidant enzymes, and four genes on the mitochondrial genome (reviewed in 1,43,44,48). Although the genes identified to date are a disparate group, some principles of adaptation are emerging. Themes include:

(a) Myosin restructuring – studies with heart of ground squirrels and hamsters suggest that changes in the proportions of myosin heavy and light chain isoforms resculpt the contractile apparatus for the new work load and thermal conditions of the torpid state.

(b) Minimizing thrombosis risk – up-regulation of α_2-macroglobulin and multiple plasma serpins, reduced platelet numbers, and reduced levels of several clotting factors all contribute to reduced clotting capacity during torpor to minimize the risk of thrombosis in the microvasculature under the very low blood flow (ischemic) conditions of the hibernating state (reviewed in 44,43). Note that this is mechanism was also seen in anoxic turtles.

(c) Mitochondrial metabolism – some genes encoded on the mitochondrial genome are up-regulated during entry into torpor including subunits of electron transport proteins (ND2, COX1) and the F_1F_0-ATPase (ATPase 6 & 8) but, significantly, nuclear-encoded subunits of these proteins (e.g. COX4, ATPα) are not altered during hibernation (22). Recall that the same phenomenon was seen under anoxia and freezing stress and this suggests that it may be a general principle of hypometabolism. Mitochondrial UCPs that function in thermogenesis are also up-regulated during hibernation.

(d) Reorganization of fuel metabolism – carbohydrate catabolism is suppressed (PDK4 phosphorylates and inhibits pyruvate dehydrogenase) and lipid and ketone body transport and catabolism are enhanced (up-regulation of FABPs, monocarboxylate transporter, lipase).

(e) Antioxidant defenses – use of nylon macroarrays revealed significant up-regulation (>2-fold) of GST, GPX and SOD in kidney and these plus Prx and metallothionein in liver (43). It has long been known that hibernators elevate AOE activities in brown adipose to deal with high rates of ROS generation during thermogenesis (3) but it now appears that this may be a general phenomenon in all organs.

In new studies, we have focused on gene/protein expression during hibernation in little brown bats, *Myotis lucifugus,* with some interesting findings. FABPs are prominently up-regulated in bat organs during hibernation (9), as they are in ground squirrels (21). The control of *fabp* gene expression is linked to the transcription factor PPARγ (peroxisome proliferator-activated receptor) and its coactivator PGC-1 which promote lipid oxidation, both of which are also up-regulated in bat organs during hibernation (8,43). Application of proteomics technology identified a new hibernation-responsive gene in bat heart, a member of the thioredoxin peroxidase family called proliferation associated gene (PAG) (10), also known as Prx 1. Two dimensional electrophoresis revealed a prominent increase in a protein with an isoelectric point of ~7 and a molecular weight of ~22 kD that was subsequently identified after tryptic digestion and mass spectrometry as PAG (Figure 1). Subsequent analysis via RT-PCR and Western blotting showed 2.3- and 4.9-fold increases in *pag* mRNA and PAG protein in bat heart during hibernation. The thioredoxin peroxidases

(peroxiredoxins) are a family of enzymes that reduce ROS in cells using thiol groups on conserved cysteine residues (positions 52 and 173) and thioredoxin as the source of reducing equivalents. As noted earlier, members of this family appear to be involved in mediating H_2O_2 levels in signalling pathways and they are up-regulated in response to stresses that elevate peroxide levels (39). Prx is also directly involved with the activation of nuclear factor kappa B (NF-κB) (27), an transcription factor that regulates gene expression, often in response to oxidative stress. With the demonstrated up-regulation of PAG (Prx1) in hibernator heart, we also wondered how markers of oxidative stress responded in hibernation. Two of these are NF-κB and the heat shock protein, HSP27. Activation of NF-κB occurs when its inhibitor protein, IκB-α, is phosphorylated at serine 32 leading to its dissociation from NF-κB, followed by ubiquitination and degradation of IκB-α. Hence, NF-κB activation status is proportional to the relative levels of phospho-IκB-α (Ser32) which increased during hibernation by 2- and 6-fold, in heart and skeletal muscle, respectively (Figure 1) (10). Similarly, levels of phospho-HSP27 rose during hibernation by 2.2-fold in heart and 3.2-fold in muscle (10).

The elevated levels of PAG (Prx1) in bat heart (10) indicate that enhanced antioxidant defenses are important in hibernation (as was also shown in ground squirrels) and this idea is further supported by our new results from microarray screening of bat skeletal muscle. Using the 19K human DNA arrays we found enhanced expression of a variety of genes in skeletal muscle of hibernating bats (Tb = 6°C) compared with aroused animals (Tb = 37°C). Binding intensities of labeled probe were 1.9-7.2 fold higher than in euthermic controls. Significantly, many of these were genes/proteins that we encountered before in hibernation or other systems of metabolic arrest. They included: (1) iron storage proteins – ferritin H and L chains, TfR2, (2) AOEs: GST A2, GPX 1 and 2, Prx 1, (3) protease inhibitors: SERPINF1 (PEDF), (4) H-FABP, the isoform in muscle and heart, and (5) several muscle motor proteins – myosin alkali light chain isoforms 4 and 6, myosin heavy chain isoforms Vc, VIIa and IXb, and actin alpha 2. The results for Prx1 and H-FABP agree with our identification of these proteins as hibernation-responsive by other means (9,10) and also validate array screening as a reliable way of finding hibernation-responsive genes. For example, four loci on the array assessed *h-fabp* and all agreed in showing 2.8-4.3 fold higher probe binding in muscle samples from hibernating versus euthermic bats; this concurs with the 1.8 ± 0.3 fold higher *h-fabp* transcript levels in hibernator muscle found by Northern blotting (9). Enhanced expression of SERPINF1 or PEDF again suggests, as discussed for anoxia tolerant turtles, that measures are taken to counteract angiogenic responses under natural states of ischemia/hypoxia that would not be improved by enhanced vascular growth. During torpor, some level of self-imposed hypoxia may occur due to the apnoic breathing patterns used by hibernators and this could trigger increased HIF-1 mediated gene transcription. Indeed, compared with euthermic ground squirrels, HIF-1α protein levels were 60-70% higher during hibernation in brown adipose and skeletal muscle, the two organs responsible for thermogenesis, and HIF-1 binding to DNA was 6-fold higher in nuclear extracts of hibernator brown adipose (34).

It is also significant that iron storage proteins and antioxidant enzymes are both up-regulated during hibernation. This emphasizes the importance of minimizing damage by ROS during hibernation and probably serves multiple purposes. One is the maintenance of long term cell viability and health during many days or weeks of continuous torpor during which strong inhibition of protein synthesis and other biosynthetic pathways (51) severely

limit the possibility of replacing macromolecules that are damaged by ROS. Another reason for good antioxidant defenses in skeletal muscle during torpor could be to minimize atrophy. Much recent evidence has implicated oxidative stress as a potential regulator of proteolytic pathways leading to muscle atrophy during periods of prolonged disuse (36). As much as possible, hibernators need to maintain their muscle mass and, without the option of exercise as a positive stimulus to counteract atrophy, the next best option may be to minimize ROS levels and the signals that stimulate atrophy. Finally, well-developed antioxidant defenses in muscle are also key to preparing for, and preventing damage from, the huge increase in ROS production that occurs within minutes when animals initiate thermogenesis and begin arousal; organ O_2 consumption can rise by 10-20 fold within minutes as the animal rewarms.

Array screening also highlighted increased levels of transcripts of selected myosin subunits and actin α2. These results suggest that remodeling of the skeletal muscle myosin motor of bats occurs during hibernation, similar to previous reports of myosin restructuring in heart of hibernating ground squirrels and hamsters (12,33). Unlike most hibernators that lie curled up in their burrows while in torpor, bats hang by their legs in their roosts and may, therefore, require a continuous level of muscle contractility. Myosin remodelling may optimize the contractile properties of skeletal muscle for two purposes: (1) to function at temperatures near 0°C during torpor, and (2) to provide effective shivering thermogenesis during arousal from torpor (initial heating is done by brown adipose but muscle shivering begins to contribute when Tb reaches ~15°C). Interestingly, two of the muscle proteins highlighted from the screening suggest that other aspects of contractile protein restructuring may also be important in hibernation. Actin α2 is a smooth muscle isoform found in the vasculature and its appearance on the list of up-regulated genes suggests that muscle restructuring for successful hibernation extends also to the smooth muscles of the blood vessels that infiltrate the skeletal muscles. Class V myosins do not participate in the contractile work of skeletal muscle but function as motors for organelle trafficking. Interestingly, overexpression studies have shown that the myosin Vc colocalizes with TfR and perturbs Tf trafficking (43). Hence, modification of iron storage or of endosome processing during hibernation may extend to a need for altered function of cytoskeletal motors.

The power of modern genomics and proteomics is impressive and is allowing us to make major advances in our understanding of how organisms adapt their metabolism to endure stresses imposed by lifestyle and environment. More importantly, the "global view" that is offered by gene screening is critical in showing us both the breadth of cellular responses and the commonalities of mechanisms that underlie organismal adaptation to environmental stress.

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LATE ABSTRACTS

103. LIVING WITH HYPOXEMIA: AN ANALYSIS OF MUSCLE SARCOPLASMIC RETICULUM PROPERTIES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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The objective of this study was to investigate the hypothesis that alterations in sarcoplasmic reticulum (SR) Ca^{2+}-cycling properties would occur in skeletal muscle of patients with moderate to severe chronic obstructive lung disease (COPD). To investigate this hypothesis, tissue samples were obtained from the vastus lateralis of 8 patients with COPD (age 67.4±2.4 yrs; FEV_1/FVC=44±2%; ±SE) and 10 healthy-matched controls (CON, age 67.5±2.4 yrs; FEV_1/FVC=77±2%) and analyzed for a wide range of SR properties. Resting PaO_2 was 83.8±2.2 mm Hg and 61.4±2.3 mm Hg in CON and COPD, respectively. As compared to CON, COPD displayed a 16% lower ($P<0.05$) maximal Ca^{2+}-ATPase activity (V_{max}, 158±vs 133±7 μmol.g protein.min^{-1}), and a 17% lower ($P<0.05$) Ca^{2+}-uptake (4.65±0.039 vs 3.85±0.26 μmol.g protein.min^{-1}) which occurred in the absence of differences in Ca^{2+}-release. The lower V_{max} in COPD was also accompanied by an 11% lower ($P<0.05$) Ca^{2+}-sensitivity, as defined by the Hill coefficient. For the Ca^{2+}-ATPase isoforms, SERCA 1a was 16% higher ($P<0.05$) and SERCA 2a was 14% lower ($P<0.05$) in COPD. It is concluded that moderate to severe COPD results in abnormalities in SR Ca^{2+}-ATPase properties, as evidenced by the dissociation between the functional and isoform phenotypes. The reduced Ca^{2+}-ATPase, which appears to be mediated by a reduction in V_{max}, suggests impairment in the ability of the muscle to relax after contraction.

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104. MONITORING HYPOXIA INDUCED ANGIOGENESIS IN BRAIN USING STEADY STATE MRI

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Angiogenesis is known to occur in brain in response to hypoxia. This study introduces an MRI method for monitoring angiogenesis in brain during hypoxic exposure using steady state R2 (STAR2) MRI, and shows the results over a time-course of 17 days exposure to ½ atm hypobaric hypoxia. Multiple measurements of CBV were made in the same animals over a time course of hypoxic exposure. Multi-echo spin echo MRI imaging was performed before and after infusion of the intravascular contrast agent, monocrystaline iron-oxide nanoparticals (MION). CBV was calculated as deltaR2t/deltaR2b, where delta=the difference in R2 before and after MION injection, t=cortical tissue and b=blood. Blood samples were obtained before and after infusion for MRI quantification of serum R2. deltaR2b was corrected for hematocrit (Hct) by deltaR2s • (1-Hct), where s=serum. CBV at day 0 measured 3.52±1.05 v/v (n=5; mean±SD). After 3 days of hypoxia, CBV significantly increased to 5.81±1.60 v/v and after 17 days CBV was 6.85±1.35 v/v. In an earlier study, we measured a CBV of 6.42±0.54 v/v (n=4; mean±SD) after 3 weeks of the same hypoxia. There was no significant difference between day 3, 17 or 21 days. Thus, after 72 hours, CBV reached a level commensurate with that obtained after 3 weeks of chronic hypoxia.This study indicates that steady-state imaging with MION is useful for quantification of CBV as a marker of angiogenesis in the same animal over a time-course study and that, within 3 days of onset of acclimation, the effective CBV has approached the final adapted volume.

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105. CLIMBING THE EDUCATION MOUNTAIN: EDUCATIONAL APPRAISAL OF THE FIRST TWO YEARS EXPERIENCE OF THE UK DIPLOMA IN MOUNTAIN MEDICINE

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30 students have now completed the diploma; we describe the candidates and course methods. Appraisal of candidates application forms, evaluation forms, and formal assessments. Description of teaching and assessment methods. Students have been from anaesthetics (34%); internal medicine (32%); or general practice (26%). They plan to use the course to help in work as an expedition doctor (47%); or in pre-hospital care (24%). A variety of teaching methods are used, including traditional lectures and seminars, but making extensive use of field work, simulations and scenario-based problem solving. A practical wilderness emergency care course has been developed and evaluated as a popular learning tool. Assessment methods include multiple choice question papers (MCQ); short answer papers (SAQ); and practical skills assessments. MCQs were difficult to write and contentious to mark, but did generate discussion and were popular for formative assessment. They were not sufficiently rigorous for summative assessment. SAQ papers were marked by two

faculty separately and the results pooled, There was good correlation between markers. Borderline papers were remarked by senior faculty. Practical skills assessment in navigation, mountain craft and wilderness medical care was by British mountain guides based upon adaptation of formal mountain qualifications, and experienced wilderness medicine practitioners in the faculty. Satisfactory assessment in both written and practical tests was required. Two candidates have been required to repeat SAQ and two subjects asked to get more experience & to present themselves for skills reassessment. Students may progress from the UIAA Diploma to a University Accredited Diploma and Masters qualification by the completion of reflective papers and a dissertation. As well as demonstrating academic rigour, these will contribute to the research base of mountain medicine.Adult learning has been successfully adapted to combine theoretical medicine & practical mountaineering skills essential for safe mountain medicine practice.

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106. INTERMITTENT HYPOXIC TRAINING TO ENHANCE ATHLETES PERFORMANCE

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The improvement of sports achievements occuring in the process of athletes training reflects the process of adaptation under the influence of means and methods used in training. Intermittent Hypoxic Training (IHT) – discontinuous normobaric hypoxia - has been used as an additional method to enhance results of endurance athletes. Twelve swimmers of high qualification were divided into two groups: control and experimental. The control group was training with usual capacity using traditional techniques. Experimental group of swimmers together with the same traditional methods of training used different variants of IHT during the recovery periods followed the main training loads. Hypoxicator 'Everest' (joint project of Climbi Ltd., Russia and Praxsep Inc., Canada) was used for creating hypoxic atmosphere. Each single hypoxic exposure was 1-5 min followed by 1-5 min 'rest' period when athletes were breathing regular air. Combined exposure did not exceed 1.5 hours within one day. The period of experimental training continued for three months. Before and immediately after the end of experiment the athletes of both groups were tested in standard ergonomic procedures for the maximum of aerobic and anaerobic work capacity. Under the conditions of the given experiment IHT resulted in significant increase in parameters of maximum aerobic capacity for the experimental group: VO_2 max increased on average 11%; Ve – 6.4%; Wer – 10.8%; pH – 44.6%; Exc CO_2 -23%, and Wmax – 9.8%. Results for the control group were significantly lower. According with these findings the greater improvement in swimming performance was to be expected for athletes in the experimental group in middle distances such as 200, 400, and 800 m. The use of IHT as additional training method for qualified swimmers made it possible to considerably improve sports results in short period of training.

107. CRF RECEPTOR TYPE 1 AND SOMATOSTATIN MODULATE PITUITARY GROWTH HORMONE AND HEPATIC INSULIN-LIKE GROWTH FACTOR-I OF RATS DURING HYPOXIA

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This study presents the responses of pituitary growth hormone (GH) and hepatic insulin-like growth factor-I (IGF-I) in rats to hypoxia and combination with cold or restraint, as well as the involvements of corticotropin-releasing factor receptor type 1 (CRFR1) and somatostatin (SS) in hypoxia-induced GH and IGF-I. Continual hypobaric hypoxia of altitude 5km (CH5km) was performed for 1 through 25d; did the combination of intermittent hypoxia of altitude 5km (IH5km) with cold (4ºC) or restraint for 2d(4h/d); CRFR1 antagonist (CP154526, 30mg/kg/d, s.c.) or SS antagonist (cysteamine, CSH, 200mg/kg/d, s.c.) were applied following exposure to 5d CH5km. Pituitary GHmRNA and hepatic IGF-I mRNA were analyzed using RT-PCR. Pituitary GH or hepatic IGF-I was measured using immunohistochemistry. During CH5km immunostaining pituitary GH (ipGH) and hepatic IGF-I (ihIGF-I) were increased for 1 and 2d, and recovered afterward; the pituitary GH mRNA was decrease on day 5, while the hepatic IGF-I mRNA reduced on day 1 and increased on day 5. IH5km, cold, restraint, and combination increased ipGH and ihIGF-I, but the restraint powerfully acted on ipGH than the IH5km or cold, and the IH5km + restraint was much potential than IH5km or IH5km + cold. Besides restraint induced a higher ihIGF-I than cold, did IH5km + restraint than IH5km, and did IH5km + cold than IH5km or cold. CP154526 and CSH significantly blocked CH5km (for 5d)-decreased pituitary GH mRNA and increased hepatic IGF-I mRNA, but remarkably induced ipGH and ihIGF-I decreases vs. CH5km + vehicle. These data suggest that CH5km could modulate the expressions of pituitary GH and hepatic IGF-I in a time-course dependent manner; restraint acts much powerfully on pituitary GH and hepatic IGF-I than hypoxia or cold, combinative stress may enlarge responses than individual one; SS and CRFR1 are involved in mediating the pituitary GH and hepatic IGF-I during CH5km.

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108. HYPOXIC ENDOTHELIN-1 EXPRESSION IN THE HEART OF MICE, OCHOTONA CURZONIAE, AND MICROTUS OECONOMUS BY COCL2-INDUCED AND HYPOBARIC HYPOXIA

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Ochotona curzoniae and Microtus oeconomus are native mammals at Qinghai-Tibetan plateau. The responses of ET-1 in the heart of these mammals to hypoxia have been unclear.In this study, both CoCl2-induced hypoxia and hypobaric altitude hypoxia (HAH) were applied. CoCl2-induced hypoxia was performed with intraperitoneal injection of CoCl2 (20, 40, and 60 mg/kg). HAH was performed in a hypobaric chamber setting at

equal to altitude of 5km (10.8%O_2) and 7km (8.2%O_2). ET-1 and HIF-1α were measured after hypoxia exposure for 6h using radioimmunoassay and Western blot, respectively. We also tested the involvement of the transcriptional factor of NF-kB in hypoxia-induced ET-1 increase through an injection (s.c.) of NF-κB antagonist.The results showed: 1. ET-1 and HIF-1a in mice heart were markedly increased intensity-dependently following an injection of three doses of CoCl2. In M. oeconomus, the increased HIF-1 α resulted from CoCl2 40 or 60mg/kg but only 60mg/kg caused ET-1 increase. However there were no changes of HIF-1α and ET-1 presented in O. curzoniae at all dosages. 2. ET-1 was markedly increased in mice heart subjecting to 5km and 7km for 6h, and 5km or 7km hypoxia-induced increased ET-1 was blocked by treatment (s.c.) with NF-κB antagonist. In M. oeconomus 5km or 7km-hypoxia caused an increased ET-1, and 5km hypoxia-induced increase was blocked by NF-κB. In O. curzoniae only 7km-hypoxia stimulated ET-1, which could not be blocked by NF-κB.These data indicate that the dramatic enhancement of ET-1 and HIF-1α in mice heart is highly sensitive to CoCl2-hypoxia or hypobaric altitude hypoxia, whereas those in O. curzoniae are entirely not sensitive compared with the mice, suggesting that O. curzoniae is well-acclimatized to environment hypoxia. Although M. oeconomus is also an inhabitant at plateau, his abilities against hypoxia are not competition with O. curzoniae. Both NF-κB and HIF-1α may be the joint transcriptional factor for ET-1 gene in mice heart, but NF-κB probably correlates with ET-1 gene in plateau mammals.

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109. CORTICOTROPIN-RELEASING FACTOR RECEPTOR 1 MEDIATES THE CONTINUAL HYPOXIA-ACTIVATED ENDOTHELIN-1 MRNA EXPRESSION IN PARAVENTRICULAR NUCLEUS OF RAT HYPOTHALAMUS

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This work aims at investigating the patterns of endothelin-1 (ET-1) changes in rat hypothalamic PVN induced by the continual hypobaric altitude hypoxia 5km (CHAH5km) and the correlation with corticotropin-releasing factor (CRF) and its receptor subtype 1 (CRFR1) in PVN activated by CHAH5km. CHAH5km (10.8 % O_2) was performed in a hypobaric chamber. ET-1 mRNA, ET-1, and CRF protein were measured using in situ hybridization and immunohistochemistry, respectively. We found that CHAH5km caused a programmed ET-1 drop and return over 1 to 25d, and the lowest point occurred on day5 of the exposure but CRF reached its peak at the same time through an early-declined phase on day 1 and 2. CHAH5km triggered ET-1 mRNA up-expression following a sharp reduction of ET-1 protein on day 5, which were completely reversed by treatment with five daily injection (s.c.) of CRFR1 antagonist (30 mg/kg/d of CP 154,526), meanwhile this treatment led to that CHAH5km-triggered increased CRF and CRF mRNA expression in PVN were also partly/completely reversed. These suggest that hypoxia-activated ET-1 release and ET-1 mRNA expression as well as CRF and CRF mRNA in PVN are mediated through CRFR1. Hypoxia activating CRFR1 in PVN positively stimulates CRF which triggers further the ET-1 release and ET-1 mRNA expression.

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110. WHOLE BODY PROTEIN TURNOVER AT HIGH ALTITUDE

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Skeletal muscle loss has often been reported as a common feature of long term exposure to high altitude. Although the muscle loss has been reported for decades the underlying regulating mechanisms are relatively unknown. We studied protein turnover in nine male subjects at sea level and after seven days exposure to 4559 m. Three days prior to experiments subjects were confined to live in our laboratory or inside the Margeritha hut at 4559 m. For this period diet and activity levels were matched. Protein turnover was studied during 4 hours of supine rest by a primed constant infusion of [1-13C]leucine and sodium bicarbonate. Blood and pulmonary CO_2 samples were obtained, before and every hour after the start of the isotope infusion. Muscle biopsies were obtained pre-infusion, and then after one and four hours. Whole body protein synthesis for the last 2 hours of the study was similar at sea level and altitude with 0.22±0.02 and 0.24±0.01 g.(kg.h)-1, respectively. Also the net whole body protein balance was similar at sea level and altitude with a net protein degradation of 0.025±0.002 and 0.030±0.002 g.(kg.h)-1, respectively. In conclusion, whole body protein synthesis and the net protein balance are unchanged after 7-9 days of acclimatization to 4559 m if activity level and diet are maintained. In addition, the previous reported altitude depended muscle loss might be related to decreased energy intake and/or activity level.

111. NEONATAL EXPOSURE TO INTERMITTENT HYPOXIA ENHANCES MICE PERFORMANCE IN WATER MAZE AND 8-ARM RADIAL MAZE TASKS

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Hypoxia has generally been reported to impair learning and memory behavior. The aim of this study was to investigate whether intermittent hypoxia (IH) might improve mice learning and memory. IH was simulated at 2 km (16.0% O_2) or 5 km (8.2% O_2) in a hypobaric chamber for 4h/d from birth to 1, 2, 3 or 4 week(s), respectively. Spatial learning and memory ability was tested in the Morris water maze (MWM) task at ages of postnatal day 36 (P36)-P40 and P85-89, respectively, and in 8-arm maze task at P60-68. The long-term potentiation (LTP), synaptic density, and phosphorylated cAMP-responsive element-binding protein (p-CREB) level in hippocampus were tested in mice at P36 under the IH for 4w (IH-4w). The results showed that IH-3w and IH-4w at 2 km significantly reduced the escape latencies of mice at P36-40 in MWM task with the significantly enhanced retention, and IH-4w-improved abilities in MWM was maintained in mice up to P85-89. At 2 km, IH-4w markedly decreased the number of error choices of mice at P60-68 in 8-arm maze

task. IH-4w at 2 km or 5 km significantly increased amplitude of LTP, the number of synapse, and the p-CREB level in the hippocampus in P36 mice. We concluded that neonatal mice subjected to IH (4h/d) at 2 km for 3w or 4w improved spatial learning and memory, which was associated with the increased p-CREB, LTP, and synapses of hippocampus in this model.

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112. THE VARIATIONS OF RESPONSIVE MANNERS BETWEEN MICE AND QINGHAI-TIBETAN PLATEAU MAMMALS TO COCL2-INDUCED HYPOXIA

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Ochotona curzoniae and Microtus oeconomus are native mammals at Qinghai-Tibetan plateau, and well acclimatized to environmental hypoxia, but the adaptive principle has not been entirely explored. This study reports the acute response of HIF-1, IGF-I, IGFBP-1, and key metabolic enzyme genes of LDH-A, ICD and SDH in liver to CoCl2-induced hypoxia. The parameters were tested 6h after CoCl2 injection i.p. (20, 40 and 60 mg/kg) to mice and plateau native mammals.The results firstly demonstrated that HIF-1a expression (transcriptional factor, steady and inducible under hypoxia) in mice was markedly increased with i.p. CoCl2 (20 or 40mg/kg), and no response in either O. curzoniae or M. oeconomus at any dose; CoCl2 significantly increased hepatic IGF-I expression in a dose-dependent manner in M. oeconomus, but not in O. curzoniae and mice; CoCl2 induced an increased hepatic IGFBP-1 in dose dependent reducing in mice, and not in M. oeconomus. In the contrast, a dramatic increasd IGFBP-1 was caused by all doses of CoCl2 in O. curzoniae, and 40 mg/kg CoCl2-induced IGFBP-1 increase maintained up to 12h (last point of observation), CoCl2 (20 mg/kg) stimulated LDH-A mRNA but reduced ICD mRNA in mice and no change in plateau native mammals.These data suggest that in contrast with lowland lab animal, plateau-acclimated native mammals are de-sensitive or high endurance to CoCl2-induced hypoxia, and maintaining the aerobic TCA may be of the characteristic manner of energy metabolism for the hypoxia-well-acclimatized plateau mammals, but the increased anaerobic LDH and reduced TCA may be the signal for triggered glycolysis pathway and/or hepatic damage for lowland mice. The IGF/IGFBP-1 systems are distinguishing for those classes, and may differentially contribute to hypoxic protection at cellular or molecular levels in the liver.

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113. EFFECTS OF SILDENAFIL ON EXERCISE CAPACITY AT THE ALTITUDE OF 5000 M ON MOUNT CHIMBORAZO

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Phosphodiesterase-5 inhibition with sildenafil has been reported to improve exercise capacity in patients with pulmonary arterial hypertension (Sastry et al, J Am Coll Cardiol 2004;43:1149-53) or with congestive heart failure (Guazzi et al, J Am Coll Cardiol 2004;44:2339-48) and in normal subjects at high altitude (Ghofrani et al, Ann Intern Med 2004;141:169-77 and Richalet et al Am J Respir Crit Care Med 2005;171:275-81). Sildenafil has been the only pharmacologic treatment until now reported to limit altitude-associated decrease in aerobic exercise capacity. We investigated the effects of the intake of 50 mg of sildenafil versus placebo in 14 healthy young adults, 6 women and 8 men, on exercise capacity as evaluated by an incremental 25 W/min cycle ergometer cardiopulmonary exercise test (CPET) at the altitude of 5000 m, in the Whymper hut, on the slopes of Mount Chimborazo, in Ecuador. Sildenafil and placebo were given on 2 consecutive days following a randomized, double blind, placebo controlled cross-over design. The subjects had been acclimatized by seven days residence at the altitude of 3800 m, with hiking at altitudes between 2800 and 4800 m. None of the subjects presented with a significant increase of the acute mountain sickness Lake Louise score at the time of the CPET. Sildenafil compared to placebo had no effect on resting O_2 saturation (81.6 ± 1.3 vs 82.7 ± 1.2 %), maximum exercise O_2 saturation (75.1 ± 1.2 vs 74.8 ± 1.0 %) resting heart rate (92 ± 4 vs 90 ± 4 bpm) or maximum workload (180 ± 15 vs 182 ± 15 W). Sildenafil had no effect on maximum O_2 uptake, maximum CO_2 ouput, maximum ventilation, ventilatory threshold, ventilatory equivalent for CO_2 at the ventilatory threshold, O_2 pulse, and maximum effort dyspnea score. The only significant changes observed after sildenafil intake were an increase in maximum heart rate (161 ± 3 vs 155 ± 4, $P < 0.05$) and a decrease in diastolic blood pressure after 2 min recovery (75 ± 4 vs 85 ± 3 mmHg, $P < 0.05$). We conclude that in the conditions of the present experiment, sildenafil did not increase aerobic exercise capacity at high altitude.

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114. OXYGEN-INDUCED PULMONARY VASODILATION IN PULMONARY ARTERIAL HYPERTENSION

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Pulmonary vascular resistance (PVR) is elevated in pulmonary arterial hypertension (PAH). Because of relatively normal alveolar oxygen tension in PAH, hypoxic pulmonary vasoconstriction should not be present and affect PVR. However, decreased mixed

venous oxygen tension ($PmvO_2$) might be of disadvantageous for the PVR. Aim: Show that increased PVR in PAH is partly due to decreased mixed venous oxygen tension, and that supplementation of oxygen can improve PVR. Methods: 16 PAH patients (10 female) underwent right heart catheterization. Mean pulmonary artery pressure and mixed venous oxygen pressure were measured. Cardiac output was calculated by the Fick method and the PVR was calculated from the cardiac output and pressure measurements. PVR was assessed during 5 minutes of breathing (1) ambient air (2) 100 % O_2 (3) 20 ppm NO and (4) 100%O_2 and 20 ppm NO. In between steps was a 5 minutes normalization period. Results: The mean PVR at baseline was 753±369 dynes.s-1.cm-5 (range: 299-1615). The overall changes in PVR from baseline to the three conditions were, (1) during NO: -24±16%, (2) during O2: -38±13%, (3) during NO and O2: -46±22%. There was no correlation between the change in PVR during NO and the change in PVR during O_2. $PmvO_2$ did not correlate with either the response of PVR to NO or the response of PVR to O_2. Even next to a vasodilator as NO, oxygen has an additive effect on the decrease in PVR in PAH, suggesting a different pathway of vasodilation by oxygen and NO. $PmvO_2$ is not predictive for the response of PVR to NO or O_2.

115. HYPOXIA AND COMBINED EXERCISE CHANGE METABOLIC ENZYME ACTIVITIES OF RAT HEART

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This study was designed to determine the effects of chronic hypobaric hypoxia solo and combined with exercise on the activity of oxidative and glycolytic enzymes. Wistar rats were divided into 4 groups: Control (C), hypoxic group (H), exercising group (E) and hypoxia-combined exercise group (HE). Rats of H and HE groups were subjected to simulated 5 000 m hypobaric hypoxia for 5 weeks (24 h/d, 7d/week), except that animals of both groups were descended to 4 000 m for 1 h/day (6 d/week) from the second week to the end, during which the HE group was forced to swim ceaselessly. Twenty four hours after the last bout of exercise training, animals were sacrificed at 4 000 m (H and HE groups) or at sea level (C and R groups), ventricles were collected and the activities of oxidative enzymes succinate dehydrogenase (SDH) and citrate synthase(CS) as well as glycolytic enzymes hexokinase (HK) and lactate dehydrogenase (LDH) were determined. SDH and CS in left ventricle (LV) were significantly lower in H than in C ($p<0.05$), but no significant difference was found between HE and C. HK, CS and LDH were significantly lower in H than in C in right ventricle (RV) ($p<0.05$), but CS and LDH exhibited no significant difference between HE and C. SDH of RV was significantly higher in HE than in C ($p<0.05$). Chronic hypoxia can diminish the activity of oxidative and glycolytic enzymes, while moderate exercise under hypoxic environment benefit to maintain these enzymes. It may be an important mechanism by which moderate exercise under hypoxic environment promote the acclimatization to hypoxia.

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116. CEREBRAL HYPOXIA INDUCES PULMONARY EDEMA: A PILOT STUDY

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High altitude pulmonary edema (HAPE), acute respiratory distress syndrome and neurogenic pulmonary edema are permeability-induced pulmonary edemas. Pathophysiological studies of these illnesses in animals have concentrated on inducing whole body hypoxia, sepsis or increasing intracranial pressure, and have neglected the influence of cerebral hypoxia alone. A series of published studies from one laboratory (Moss, 1968-1972) suggests a link between pulmonary edema (PE) in an animal model where the brain is hypoxic in the presence of systemic normoxia. These studies have not been replicated. Thus, we hypothesized that cerebral hypoxia (CH) coupled with whole body normoxia would induce pulmonary edema in mongrel dogs. Methods: Indices of pulmonary edema, hemodynamic parameters and norepinephrine (NE) values were measured in anesthetized adult Walker hounds in which one carotid artery was ligated and the other delivered a venous (n=3) or arterial (n=1; CTRL) perfusate. In CH PO_2, PCO_2 and SaO_2 were maintained for 2 h at 38±1, 44±1,and 63±7 vs. 83±4, 40±1, and 94±1 in CTRL, while the systemic PO_2, PCO_2 and SaO_2 were maintained as in normoxia. After 2 h of CH, normoxic cerebral blood perfusion was reinstated and dogs breathed room air spontaneously for an additional 2 h before euthanasia. Results: Lung wet weight-to-dry weight ratios were increased in the CH group compared to CTRL (6±1 vs. 2). Histology revealed areas of marked increases of neutrophils and macrophages, acute hemorrhage, congestion and alveolar edema in the CH animals, but not CTRL. In CH arterial PO_2, SaO_2 and pH decreased to 42±3, 63±4 and 7.22±0.02 respectively during the 2 hr of "recovery" while PCO_2 increased to 51±2 mm Hg. There were no significant differences in mean arterial, pulmonary wedge and pulmonary artery pressures or cardiac output between the CH and CTRL. However, NE increased 5-fold more in the CH vs. CTRL animal. Discussion: CH increases NE and induces PE. We speculate that the increase in permeability may be directly modulated by NE, however other hypoxia-initiated factors may be involved. For example, neuropeptide-Y is often elevated in conjunction with elevated NE, and NPY can cause increases in vascular permeability. Further research is necessary to answer these questions, and to establish if this model can serve as an animal model for HAPE. It may be that the cerebral hypoxia induced in our model activates a series of pathophysiological responses similar to those encountered by the apneic, hypoxemic climber sleeping at very high altitudes. In that case, the brain is certainly hypoxic and its contribution to HAPE may be explored using the cerebral hypoxia model.

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117. LAKE LOUISE SCORING SYSTEM UNDERESTIMATES SYMPTOMS OF ACUTE MOUNTAIN SICKNESS IN 4-11 YEAR OLD CHILDREN

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The Lake Louise Scoring System (LLSS) is a self-reporting tool designed to evaluate adults for symptoms of acute mountain sickness (AMS). Our objective was to evaluate if age appropriate language alters the results of the AMS diagnostic score in 4-11 yr old children. In this prospective study, subjects completed both the LLSS and an equivalent modified language symptom score (MLS) once daily for 3 days. The MLS included an additional "faces pain scale" to quantify headache symptoms. Parents were allowed to assist children with questions on both surveys. Measurements were made at 1605m, in the subject's homes, without any altitude change. Equivalent symptom questions between the two surveys were assessed for agreement on the day when the most symptoms were recorded for each question. Thirty-seven children (19 girls), ages 4 to 11 (mean age 7.38 ± 2.28 years) completed the study. Six children enrolled were lost to follow-up. Kappa values: headache (K=0.22), gastrointestinal (K=0.34), fatigue (K=0.88), dizziness (K=0.65) and sleep (K=0.88) ranged from fair to very good. The MLS resulted in higher mean symptom scores (1.14 ± .98) compared to LLSS questions (.61 ± .82) ($p<.01$). The AMS diagnostic threshold, including both a headache and a total score of 3 or greater, was reached in 9% (95%CI, 4-16) of measurements using the MLS and 4.5% (95%CI, 1.5-10) with the LLSS. The LLSS underestimates symptoms associated with AMS in 4-11 year old children. Age appropriate language must be used in the development of any AMS diagnostic tools; particularly for headache (the key symptom of AMS) and gastrointestinal symptoms. After normal values are established, the numerical threshold for AMS diagnosis may require modification in this age population.

118. FOOD CHOICES FOCUSING ON CARBOHYDRATE AND FAT CONSUMPTION AT FIVE ALTITUDES ON A CLIMBING EXPEDITION TO MOUNT EVEREST

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The initial field study was designed to determine the overall consumption of energy, the distribution of the macronutrients which provided the energy, and the effects of increasing altitude on total energy consumption and on changes in energy-providing macronutrients. The 9-week dietary study included three meals and daily snacks on a 10-day rotation. A detailed record of energy intake was kept by five subjects who remained in base camp (5300m) and by 10 subjects who climbed to altitudes up to and including the summit of

Mt. Everest (8848m). Free choice of individual items and amounts within the diet was permitted. Intake of food and fluid was determined by means of monitored entries in 843 daily food records. It is commonly assumed that there is a natural increase in preference for consumption of higher carbohydrate foods at the highest altitudes. Contrary to these beliefs, the climbers did successfully self-select an average of 28.5% fat, 55.5% carbohydrate and l4.5% protein. A practical outgrowth of the study was the derivation of food frequency lists itemizing the top 25 choices in descending order for the five camps. Energy intake percentages for fat and carbohydrate were also determined for each of these 25 food selections. Upon reviewing the reported food items, it appears that higher fat foods should not be excluded from the packs of climbers going to higher altitudes. Energy-dense foods may be encouraged to provide extra energy and alleviate loss of weight.

119. THE EFFECT OF CARBON DIOXIDE ON CEREBRAL AND SKELETAL MUSCLE TISSUE OXYGENATION RESPONSES DURING ACUTE HYPOXIA

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This study was conducted to test the hypothesis that during hypoxia, hypercapnia would increase mean arterial blood pressure (MAP), minute ventilation (VE), and maintain cerebral tissue oxygenation (cTOI) to a greater degree than normocapnia. A secondary purpose was to explore the effect of our intervention on skeletal muscle tissue oxygenation (mTOI). Eight healthy males completed two hypoxic protocols (SaO_2=80.3 ± 0.7%, mean ± SEM), defined by either increased (+5 Torr) or eupnic end-tidal PCO_2 ($PETCO_2$). Each protocol was delivered in random order on two separate days and consisted of five segments: three 10 min resting periods separated by a hypoxic ventilatory response test (HVR) and then a 20 min hypoxic exposure. Near infrared spectroscopy was applied to the left cerebral cortex and vastus lateralis muscle. During the 20 min hypoxic exposure, a trend towards a greater reduction in cTOI was observed for normocapnia compared with hypercapnia (8.2 ± 0.8 vs. 6.2 ± 0.8 %, p=0.08). A main effect for time was observed during the hypoxic exposure, showing a progressive reduction in cTOI ($p<0.01$) which was greater during the hypercapnic protocol (p=0.05). The reduction in mTOI was not significantly different between each protocol, however, a main effect for time showed that mTOI progressively decreased ($p<0.01$). The HVR values were not different between protocols (0.74 ± 0.1 L min-1 % SaO_2^{-1}, range: 0.21-1.23). Throughout the hypoxic exposure, hypercapnia compared to normocapnia resulted in greater increases in MAP (8.3 ± 1.4 vs 3.8 ± 1.4 mmHg, p=0.04) and VE (18.1 ± 2.9 vs 4.3 ± 2.9 L min-1, $p<0.01$).When hypoxia is combined with hypercapnia, cTOI is maintained to a greater degree than during normocapnia, a likely result of CO_2-mediated cerebral vasodilatation. The mTOI demonstrated no differential effect between protocols, and therefore the skeletal vascular responses do not appear to be modulated by CO_2 in the same way as the cerebral vasculature.

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120. ESTIMATING VA FROM FRESH GAS FLOW AND END-TIDAL FCO2 USING A SEQUENTIAL REBREATHING CIRCUIT

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To maintain isocapnia using a sequential rebreathing circuit, fresh gas flow (FGF) must be set equal to alveolar ventilation (VA). Simple observation can identify when FGF is equal to minute ventilation (VE), but not when it is equal to VA. We devised and validated a method of setting FGF to VA.Seven healthy subjects breathed via a commercial sequential rebreathing circuit supplying fresh gas and previously exhaled gas in sequence. Initially, FGF was set at approximately equal to VE. Minute CO_2 production (VCO_2) was calculated as the product of the FGF and the fractional concentration of CO_2 in the exhaled gas reservoir. The VA was calculated as VCO_2 divided by the end-tidal fractional CO_2 concentration ($FETCO_2$). FGF was decreased to the calculated VA for a control period, following which subjects were asked to increase their ventilation. FGF was confirmed to be equal to VA if $FETCO_2$ did not change with marked hyperpnea.$FETCO_2$ did not change significantly from control breathing (41.22 ± 0.39 mmHg) to hyperventilation (40.89 ± 0.27 mmHg) ($p = 0.820$), despite increases in VE from 11.16 ± 2.70 L/min to 40.30 ± 2.68 L/min ($p < 0.001$). When breathing via a sequential rebreathing circuit, VA can be approximated from FGF, $FETCO_2$ and CO_2 concentrations in mixed exhaled gas, all of which are readily measurable parameters. The result is sufficiently accurate to maintain isocapnia at increased minute ventilation.

121. POSSIBLE ROLE OF LUNG VOLUMES, ALVEOLAR PO2 AND PCO2 ON BREATH HOLDING TIME

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To evaluate the effect of lung volume, alveolar hypoxia and hypercapnia on breath holding time (BHT), 10 healthy subjects participated in this study. They conducted breath holding (BH) starting from three different lung volume, i.e., TLC, FRC and RV (air trials: TLC-air, FRC-air and RV-air). In addition, to cancel alveolar hypoxia due to progress of BH, subjects started BH following 100% oxygen inhalation (oxygen trials: TLC-O_2, FRC-O_2 and RV-O_2). Furthermore they also performed BH from TLC right after inhalation air or 100% oxygen containing 5% CO_2 (CO_2 trials: TLC-airCO_2, TLC-O_2CO_2). Therefore, each subject challenged eight BH trials. $PETO_2$ and $PETCO_2$ at breaking point (BP) were also monitored. Mean BHT in TLC-air, FRC-air and RV-air were 86.5, 40.0 and 30.4 sec, respectively. And mean BHT in TLC-O_2, FRC-O_2 and RV-O_2 were 123.3, 84.4 and 45.3 sec, respectively. In all O_2 trials, $PETO_2$ at BP were higher than 400mmHg with higher $PETCO_2$ at BP than air trials. From these results, we confirmed that BHT was prolonged in accordance with starting lung volume and determined by combined effect of alveolar hypoxia and hypercapnia. However, when BHT was plotted against actual lung volumes,

we found dogleg-like relationship between these two variables, which was not linear. On the other hand, when we cancelled alveolar hypocapnia by breathing mixed gases containing 5% CO_2 in TLC trials (CO_2 trials), BHT became shorter and the dogleg shape disappeared. Correlation of BHT with lung volume became linear significantly.These results suggest that a part of prolongation of BHT from TLC is explained by initial low alveolar hypocapnia, which is cancelled by 5% CO_2 inhalation, induced by CO_2 dilution by deep breath at TLC.

122. GASTRIC TONOMETRY AT REST AND DURING EXERCISE AT 5100M

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To determine the effect of exercise on intragastric CO_2 levels, measured using a gastric tonometer, at 5100m. Background: Reduced gastric perfusion (indicated by abnormal gastric CO_2 tonometry) is associated with an adverse outcome in critically ill patients(1). Location: Chamlang basecamp (5100m), Hongu Valley, Solu Khumbu, Nepal. Subjects: 5 well acclimatised healthy male volunteers. Preparation: Ranitidine 150mg 12 and 2 hours prior to study, nil by mouth 12 hours prior to study. Intervention: Stepping on and off 0.2m step, 6 Kg backpack, 30 minutes rest, 15 minutes at heart rate 110 (HR110), 15 minutes at heart rate 140 (HR140), 15 minutes rest. Measurements: Heart rate and oxygen saturation (Novametrix model 513 pulse oximeter), end-tidal(et)PCO_2 and gastric(g)PCO_2 (TONOCAP, Datex-Ohmeda, Finland). Gastric/end-tidal PCO_2 gap (P(g-et)CO_2) was calculated by subtracting gastric PCO_2 from end-tidal PCO_2 at each time point. Statistical analysis: Mean values were compared using 2 tailed paired t-tests. All subjects (Age range: 30-38) completed the protocol. Mean oxygen saturations: When compared with rest (SpO_2 91.2) HR110 (SpO_2 79.3, $p<0.05$) and HR140 (SpO_2 80.7, $p<0.05$) were reduced. Mean gastric/end-tidal CO_2 gap: When compared with rest (P(g-et)CO2 0.22) there was no increase at HR110 (P(g-et)CO_2 -0.18, NS) but a significant increase at HR140 (P(g-et)CO_2 0.77, $p<0.05$).These are the first data demonstrating that intragastric PCO_2 during exercise in a hypoxic environment falls relative to end tidal PCO_2 suggesting that gastric perfusion may be reduced.

Reference

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123. MODIFIED OXYGEN MASK TO INDUCE TARGET LEVELS OF HYPEROXIA AND HYPERCARBIA: A MORE EFFECTIVE ALTERNATIVE TO CARBOGEN

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Carbogen (95-98% O_2, 5-2% CO_2) inhalation during radiotherapy is designed to enhance tumor radiosensitivity by increasing cellular PO_2. CO_2 is administered to increase tumor perfusion. In practice, however, carbogen is inconsistent in raising PCO_2. We investigated a partial rebreathing method to raise PCO_2, and compared its efficacy to that of carbogen. Ten healthy volunteers breathed 1.5, 3 and 5% carbogen in 5 minute stages via a non-rebreathing circuit. At the end of the 5% carbogen stage, four volunteers voluntarily hyperventilated. All the volunteers then breathed 100% oxygen through a commercial sequential gas delivery oxygen mask modified by attaching a reservoir to its exhalation port. Oxygen flow to the circuit was reduced to induce hypercapnia. At the end of the study, the same four subjects voluntarily hyperventilated. In all experiments minute ventilation and end-tidal PCO_2 ($PETCO_2$) were measured at steady-state. $PETCO_2$ did not increase from baseline (40±1.5 mmHg) when 1.5 ($p=0.26$) and 3% carbogen ($p=0.38$) were inhaled. Breathing 5% carbogen increased $PETCO_2$ to 45±1.6 mmHg ($p<0.001$); however, voluntary hyperventilation reduced $PETCO_2$ back to control levels ($p=0.987$). With the sequential gas delivery method, reducing O2 flow to 4.3±0.7 L/min increased $PETCO_2$ from 41±2.0mmHg (baseline) to 46±2.1 mmHg ($p<0.001$). In contrast to carbogen, however, voluntary hyperventilation at the same O_2 flow did not reduce $PETCO_2$ ($p=0.379$). Precise $PETCO_2$ can be reliably induced and maintained by controlling O_2 flow into a modified sequential gas delivery system. We suggest that this is a simpler and more reliable means than carbogen, of raising arterial PCO_2 during radiotherapy.

124. LOW BIRTH WEIGHT IN COLORADO: ANALYSIS USING GEOGRAPHIC INFORMATION SYSTEMS

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The rate of low birth weight (< 2500 grams) in Colorado consistently exceeds the national average. Analyzing risk factors for low birth weight at altitude has been difficult due to the inability to precisely define the elevation of the mother during pregnancy. The use of geographic information systems (GIS) in the analysis of low birth weight in Colorado results in greater accuracy in altitude assignment for births, new methods for examining the interaction of altitude with social and medical factors, and enhanced graphic representation of results as compared with previous analyses. To advance understanding of the interaction

between the environment (altitude) and potentially modifiable health and behavioral factors, 560,840 birth records from 1993-2002 were assigned latitude, longitude, and accurate elevation based on the mother's residence. Statistical tools were used to detect significant clusters of events (SatScan and spatial statistics tools found in ArcGIS 9.0). Clusters of low weight births and maternal smoking were identified both within prescribed ranges of altitude and across all altitudes. ArcGIS software was then used to create maps that linked altitude, demographic, and health services data.GIS analysis offers unique advantages for the study of altitude-related health conditions.

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125. EFFECTS OF SPRINT INTERVAL TRAINING UNDER NORMOBARIC NORMOXIA AND HYPOXIA ON MYOSIN HEAVY CHAIN COMPOSITION AND BIOENERGETIC PROPERTIES IN RAT DIAPHRAGM

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The purposes of the present study were to examine 1) whether sprint interval training (SIT) could alter the myosin heavy chain (MyHC) composition and bioenergetic properties of rat diaphragm, 2) whether hypoxia could enhance the effects of SIT on rat diaphragm. The present study was approved by the Juntendo University Animal Care and Use Committee. Male Wistar rats (7 weeks old) were divided into 3 groups with matched weight, control: CON (n=7), normoxic training: NT (n=7), hypoxic training: HT (n=7). SIT (1 min/sprint, 6-10 repetitions/day and 5-6 days/week for 9 weeks) was conducted on an animal treadmill under oxygen concentration of 20.9% for NT and 14.5% for HT. The treadmill speed was 75-80 m/min at the final week. Costal diaphragm portions were removed 48 hours after the last training session to analyze MyHC composition (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and citrate synthase (CS) and lactate dehydrogenase (LDH) activities (spectrophotometry). In MyHC composition, type IIa isoforms were increased (CON: 20.5 < NT: 24.6 < HT: 30.1%, $P < 0.05$) and type IId isoform were decreased (CON: 51.7 > NT: 47.2 > HT: 42.1%, $P < 0.05$) by SIT. Thus, hypoxia resulted in greater changes of MyHC composition. CS activities were increased (CON: 293 ± 31 < NT: 342 ± 38 < HT: 391 ± 15 nM/min/mg protein, $P < 0.05$) by SIT, and the activity of HT was significantly higher than NT ($P < 0.05$). But, LDH activities were decreased by SIT. SIT could induce the increasing of oxidative capacity and the fast-to-slow shift of MyHC composition in diaphragm, which would contribute to improve the diaphragmatic endurance ability. In addition hypoxia could enhance such improvements.

126. NON-INVASIVE MEASUREMENT OF CARDIAC OUTPUT DURING SPONTANEOUS BREATHING

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All respiratory-based methods of measuring cardiac output depend on imposing a step-change in alveolar ventilation (VA) for a particular gas. For CO_2, this can be accomplished by interposing respiratory deadspace in the breathing circuit, but therefore requires a uniform breathing pattern. We developed a method of reducing VA that is independent of breathing pattern using a new breathing circuit. We evaluated this method's accuracy by comparing its measurements (QNI) with those of thermodilution (QTD) on cardiac patients in the ICU, as well as its precision by performing repeated tests on subjects in a laboratory. Our subjects consisted of 31 extubated post cardiac surgery patients in the cardiovascular intensive care unit. The reduction in VA was induced by a step reduction in gas flow to the circuit. The balance of ventilation was composed of previously exhaled gas and no respiratory manoeuvre was required by the patient. QTD was measured simultaneously. We also conducted measurements to evaluate precision by performing 8 repeated tests on 24 subjects in a laboratory setting. In our validation study, QNI was highly correlated with QTD (R2 = 0.89) despite a wide range of breathing patterns. The bias and precision of the non-invasive method were -0.08 and 0.80 L/min, respectively. The repeatability of QNI, measured as the SD of repeated tests, was 0.73 L/min, which is slightly less that the repeatability of QTD, which is usually reported as 0.60 L/min.We have developed and tested a new method for measuring cardiac output non-invasively in spontaneously breathing subjects. Although measures to improve accuracy are still needed, our method correlated well with thermodilution and may eventually provide researchers with a new, clinically useful, non-invasive test.

127. HIGH ALTITUDE PULMONARY EDEMA FOLLOWING MARIJUANA SMOKE INHALATION

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Association between cannabis smoke and high altitude pulmonary edema (HAPE) has not been described. This is a case report of HAPE in a non-smoker following inhalation of marijuana smoke. We believe marijuana may modulate hypoxic pulmonary vasoconstriction. A healthy, non-smoking 26 year-old male participated in a high altitude expedition. The ascent profile allowed for acclimatization. At 2550m and having asymptomatically been to 3100m he smoked a large quantity of marijuana. He developed dry cough, exertional dyspnoea and was disproportionately tired. He continued with the trek that day crossing a pass at 3050m, descending to camp at 2800m. There he subjectively worsened. On examination he was apyrexial, alert, with pulse 102min^{-1}, blood pressure 130/88mmHg, respiratory rate 18min-1, bilateral basal crepitations and finger plethysmography oxygen

saturation 89% falling to 79% on minimal exertion. HAPE was diagnosed. The treatment was descent by 600m, nifedipine (SR 20mg tds), azithromycin and acetazolamide (250mg bd). Following rest, taking nifedipine and acetazolamide, he uneventfully continued the ascent to 5100m. Pulmonary Doppler showed significant increase in pulmonary arterial pressure in spite of nifedipine. It is known that marijuana does not cause severe changes in pulmonary capillary permeability [1] though chronic use may cause airway inflammation [2]. It is possible that marijuana smoke has an effect on hypoxic pulmonary vasoconstriction (HPV). Animal studies suggest smoking can augment HPV [3]. Effect of smoking and cannabis use on HPV in humans deserves further studies.

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128. EXPRESSION OF PROTEINS OF IRON METABOLISM IN HUMAN SKELETAL MUSCLE AT HIGH ALTITUDE

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Hypoxia enhances the synthesis of several hemoproteins. In cell lines exposed to hypoxia, transferrin receptor (TfR) – a membrane protein that controls cellular iron uptake and contributes to heme synthesis - was previously found upregulated. Other iron-related proteins were also found modulated by hypoxia. However, the regulation of these proteins during hypoxia at the muscle level is unknown. The present study therefore evaluated the effect of hypobaric hypoxia on the expression of proteins involved in iron metabolism in human skeletal muscle. Nine male lowlanders were evaluated at sea-level (SL) and after 7-9 days spent at high altitude (HA, 4,559 m). Soluble TfR (sTfR), ferritin and erythropoietin (EPO) were determined in venous serum by standard methods. Muscle biopsy specimens were obtained from the vastus lateralis to assess the expression of TfR by immunoblot analysis, H and L ferritin subunits by ELISA, Iron Regulatory Protein (IRP) binding activity by bandshift analysis and Hypoxia Inducible Factor (HIF)-1α; and -2α; mRNAs by real-time RT-PCR. Serum analysis showed that HA induced a two-fold increase in sTfR levels, a significant increase in EPO and a strong decrease in ferritin concentrations. Analysis of muscle samples revealed that both HIF-1α and HIF-2α mRNAs levels were 2-3 fold higher in HA. Surprisingly, TfR content was diminished by 50 % (average decrease) in HA, along

with a decreased IRP binding activity. HA did not alter the content of H ferritin subunits, but decreased the content of L ferritin subunits. Expression of iron-related proteins was decreased in skeletal muscle during prolonged hypoxia, at a time when erythropoiesis was enhanced. This unexpected response into the muscle tissue suggests that the strong iron demand associated with high erythropoiesis would trigger a mobilization of muscle iron stores.

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129. RELIABILITY OF INDUCTION OF HYPOXIA FOR HYPOXIC TRAINING WITH A NEW CIRCUIT COMPARED TO THAT WITH ALTIPOWER

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Intermittent hypoxic training has been shown to enhance athletic performance and high altitude acclimatization. However, efficacy of a training device may be limited by the reliability of induction of hypoxia. We devised a semi-closed breathing circuit designed to reliably attain and maintain target levels of $FETO_2$ independent of increases in minute ventilation. The purpose of the study was to compare the performance of our circuit to a popular commercial hypoxic training device (Altipower) that also does not require any electronics or compressed gases. Five subjects participated in the study. Each subject breathed through Altipower at each of the 4 settings set by the manufacturer, for 5 min and then hyperventilated for 1 min. Subjects breathed room air at rest between each test. The same protocol was followed while breathing via the new circuit. $FETO_2$ and SpO_2% was measured continuously. In our circuit, the precise flow of ambient air was set at 60% of the participant's alveolar ventilation (as estimated by the Radford nomogram) for the first level, followed by 3 further reductions of approximately 5% of VA. With the Altipower there was no consistent reduction in $FETO_2$ at any of the settings. In some subjects there was no reduction and in others the reductions were to $FETO_2$ less than 6% (at levels 3 and 4), which the subjects found intolerable. Hyperventilation returned the $FETO_2$ to control in the majority of the subjects. With our circuit the reductions in O_2 were consistent from subject to subject (FETO2 were within a small range) and were further reduced at each incremental setting. Hyperventilation had little effect on $FETO_2$ (changes were less than 1% at all settings). Our hypoxic circuit allows reliable setting of $FETO_2$, which is well maintained even with hyperventilation.

130. THE PROFILE OF HYPOXIA EXPOSURE INFLUENCES THE RELATIONSHIP BETWEEN BLOOD PRESSURE & INOS RESPONSE

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Determinants of the nature and magnitude of the hemodynamic responses to hypoxia may include the specific profile of exposure and variation in the expression of relevant genes such as those responsible for inducible nitric oxide synthase (iNOS). The purpose of this investigation was to examine the relationship between blood pressure (BP) and iNOS mRNA to daily comparable total exposure time of intermittent hypoxia (IH) vs. continuous hypoxia (CH). Ten normal males had six 10-min. hypoxic exposures (oxyhemoglobin saturation [SpO_2] = 80-90%) separated by 10 min. of normoxia on 3 consecutive days. Subjects also had 3 consecutive days of CH (60 min/day; SpO_2: 80-90%). A washout period of >7 days separated IH and CH exposure blocks. Heart rate, BP, SpO_2, and $ETCO_2$ were recorded during the 5 min. prior to the start of each day's IH and CH session, as well as during the last 5 min. of hypoxia exposure on each day. Venous blood for iNOS mRNA was obtained (using PAXgene Blood RNA System) before IH and CH exposure on day 1, and 2 hours after the last exposure on day 3. The relationship between the hemodynamic and molecular parameters was determined via nonparametric correlation. Usable data for analysis were available on 9 subjects. At the end of the day 3 IH session, there was a significant negative correlation ($p<0.01$) between both diastolic and mean BP with iNOS mRNA. The negative correlation between BP and iNOS mRNA in conjunction with IH, but not CH, exposure suggests that the hemodynamic response to IH, but not CH, may be modulated by iNOS. The inter-individual differences in the iNOS/BP relationship during IH may provide further insight into hypertension development in humans undergoing regular episodic hypoxia exposure.

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131. SENSITIVITY ANALYSIS OF THE FACTORS INFLUENCING VO2,MAX IN BIRDS DURING NORMOXIA AND HYPOXIA

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The objective of this study was to examine how various factors may influence maximal oxygen consumption in birds during normoxia, moderate hypoxia, and severe hypoxia. In doing so, we hope to reveal the traits that may be adaptive in high altitude species. We used a theoretical approach involving a numerical model of three equations to calculate oxygen consumption, namely mass conservation across the lungs and a version of the Fick equation at both the lungs and tissues. Physiological traits were then varied to determine the relative influence each has on VO_2max. Of those traits that were analyzed, increasing the

diffusional conductance of the tissue for O_2 (DT) had the greatest influence on VO_2,max for all environmental oxygen concentrations, while conductance at the lungs (DL) had a much more modest effect. In normoxia, low haemoglobin (Hb) oxygen affinity (high P50), high Hb-O_2 cooperativity (Hill coefficients above 2.8), and high Bohr shift all had beneficial effects on oxygen consumption. In contrast, severe hypoxia favoured a lower P50 and less cooperativity, while a pronounced Bohr shift had less influence. Ventilation rate, cardiac output, and Hb concentration all had a greater influence on VO_2,max during severe hypoxia, but only ventilation had appreciable effects. Perhaps most interesting was the interaction between P50 and DT: at lower P50, DT had a much greater influence on oxygen consumption at all environmental oxygen concentrations. Indeed, the greatest relative increases in VO_2,max were always observed when P50 decreased and DT increased concurrently. These results suggest that high Hb-O_2 affinity increases the selective advantage of high tissue O_2 conductance, and that concurrent changes in both P50 and DT may be required for performance at high altitude in birds.

132. GENDER-SPECIFIC EFFECT OF NMDA GLUTAMATE RECEPTORS ON RESPIRATORY AND METABOLIC ADAPTATION TO CHRONIC HYPOXIA IN NEWBORN RATS

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Newborn mammals exposed to chronic hypoxia develop several mechanisms including metabolic and ventilatory adjustments, but the neural mechanisms involved in these modifications are not known. Since previous studies have reported that the expression of glutamatergic NMDA receptor is drastically upregulated during chronic hypoxia in rat pups, we tested the hypothesis that the function of NMDA receptors on respiratory and metabolic homeostasis is altered in newborn rats raised under chronic hypoxia. Respiratory and metabolic recordings were performed in 10 days-old rat pups (P10) raised from one day before birth in chronic hypoxia (CHx: 12-13% O_2) or normoxia (Nx: 21% O_2). Using whole body plethysmography, we recorded minute ventilation (Ve-mL/min/100g), Rectal Temperature (Tb-°C) and oxygen consumption (VO_2 -mL/min/100g), 30 minutes after intraperitonial injection of vehicle or NMDA receptor antagonist MK-801 (1mg/kg). Recording chamber was maintained at 32°C. Recordings were performed in males and females under conditions used during growth (CHx or Nx).CHx pups had lower body weight (males -29%; females -27% - both $p<0.0001$) and Tb (males -0.5 °C ; females -0.9°C - both $p<0.0001$) than Nx. Hypoxic male and female pups had similar VO_2 than normoxic, while Ve was increased in CHx males (+40% - $p=0.001$) but not in CHx females (+2% - p=ns). In normoxic rats MK-801 had no effect. In hypoxic males, after MK-801 injection Tb, VO_2 and Ve dropped (Tb: -0,9°C - $p<0.01$; VO2: -21% - $p<0.02$; Ve: -20% - $p<0.05$ vs. saline) while in females only Tb dropped significantly (-0.9 °C - $p<0.01$; VO_2: -13% - p=ns; Ve: +18% p=ns).Male rat pups raised in hypoxia relied on NMDA glutamate receptors function to maintain VO_2, Ve and Tb at an optimal level.

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133. CEREBRAL BLOOD FLOW AND METABOLISM IN SEVERELY HEAD-INJURED PATIENTS WITH HYPOTHERMIA

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The effect of therapeutic hypothermia for head injury is controversial. Clifton mentioned the impact of hypothermia on admission in severely head-injured patients (N Engl J Med 2001 Feb). We compared severely head-injured patients of accidental hypothermia (AH group) and those who were not hypothermia on admission and treated by therapeutic hypothermia (TH group) by measuring cerebral blood flow (CBF) and cerebral metabolic rates for oxygen (CMRO2). In five cases of acute subdural hematoma (Glasgow Coma Scale scores of 6 or less on admission), CBF and $CMRO_2$ were measured within 5 days after injury by Kety-Schmidt technique using N_2O as the indicator. Three cases of AH group and 2 cases of TH group were all treated with mild hypothermia (33-34degrees Celsius) after evacuation of hematoma. Three cases except for moderate hypothermia cases were sedated with Midazolam during cooling. Outcomes were determined at discharge using Glasgow Outcome Scale.In AH group, two cases of moderate hypothermia on admission (29.5-31degrees Celsius) showed extremely low CBF (15.4 and 6.9ml/100g/min) and $CMRO_2$ (0.17 and 0.21ml/100g/min), and both of them died. In the rest of AH group, the case of mild hypothermia (34.5degrees Celsius), CBF was 32.9 and $CMRO_2$ was 2.87ml/100g/min, and outcome was SD. In TH group, CBF were 29.4 and 49.3 ml/100g/min, CMRO2 were 1.48 and 2.52ml/100g/min, and outcomes were SD and MD.

134. NEW CHEMICAL OXYGEN GENERATOR TESTED IN MT. FUJI

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In Japan, 5.6 million people enjoy mountaineering. So, we (the members of Japaense Society of Travel Medicine) give them practical safety advice of mountaineering, including of AMS (Acute Mountain Sickness) prevention and self-treatment. We tested the effectiveness of New Chemical Oxygen Generator (O_2 Forest, ACS Co.Ltd.,Tokyo,Japan) in Mt. Fuji (3,776m).We measured oxygen saturation of 33 healthy volunteers at the hight of 2,500m, 2,700m, 3,000m, 3,300m, 3,600m, 3,776m, using portable pulse-oxymeter (FO-0001-3A, Casio Co.Ltd.,Tokyo, Japan). After each measurement, low flow oxygen (0.4 L/Min.), generated by New Chemical Oxygen Generator, was inhaled for 3 minutes, and oxygen saturation was again measured and recorded. Oxygen saturation average values without oxygen inhalation are as follows: 93.2%(2,500m), 92.9%(2,700m), 92.3%(3,000m), 83.6%(3,600m), 81.4%(3.776m). Oxygen saturation after 3 minutes oxygen inhalation are as follows: 93.7%(2,500m), 93.5%(2,700m), 93.6%(3,000m), 92.2%(3,300m), 91.6%(3,600m), 90.7%(3,776m). New Chemical Oxygen Generator enhances oxygen saturation at the height from 2,500m up to 3,776m in Mt. Fuji. So, this new portable oxygen

generator could be an excellent modality for the treatment of mild and moderate case of AMS.

Acknowledgements: 33 sets of portable oxygen generators were donated from ACS Co.Ltd. Japanese Society of Travel Medicine Resarch Fund was given to this project.

135. CEREBROVASCULAR RESPONSE TO ULTRA-SHORT CYCLIC ALTERATIONS IN END-TIDAL PCO2

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Assessment of cerebrovascular reactivity with functional MRI requires rapid cycling of end-tidal PCO_2 ($PETCO_2$) between two steady-states. To optimize signal to noise ratio we devised a method of iso-oxic cycling between $PETCO_2$ of 30 and 40 mmHg with 15 s transitions and steady state (<2 mmHg variation in $PETCO_2$) of 30 s at each level (rapid cycling). We used trans-cranial Doppler to confirm that the protocol provides sufficient time for full cerebrovascular response. In addition, we tested whether there is hysteresis or fatigue of response with repeated cycles. We alternated 'rapid cycles' with periods of 'slow cycles' in which the steady state components were prolonged to 2 min but transition rates remained unchanged. The protocol was carried out for 15 min in 4 subjects. Time constants for the rising MCAV were the same in fast and slow cycles, as were those of the falling MCAV. MCAV reached the same levels at each respective 'steady state' phase of the rapid and slow cycles. MCAV response to each level of $PETCO_2$ did not change over the course of the experiment. Rapid square-wave cycles with steady-state phases as short as 30 seconds exhibit full MCAV response to changes in $PETCO_2$ without fatigue or hysteresis. We speculate that similar results could be expected with the regional blood flow as measured by MRI.

136. HOW HIGH CAN YOU GET: RAISING THE ANTE ON OXYGEN DELIVERY

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Non-rebreathing (NRM) and Venturi O_2 masks cannot consistently provide high inspired fractional concentrations of oxygen (FIO_2) to breathless or severely hypoxic patients. We compared the performance of these masks to a new O_2 mask (HiOx80) that exploits efficiencies afforded by sequential gas delivery. Eight volunteers breathed through each of the masks at rest and at 10, 14, 18, 24 and 30 L/min. O_2 flow to the HiOx80 mask was 2, 4 and 8 L/min, but 8 L/min to the other masks. We used end tidal partial pressures of O_2 and CO_2 and the alveolar gas equation to calculate FIO_2.The HiOx80 was the only mask to provide $FIO_2 > 0.8$ and it consistently provided the highest FIO_2 among the three masks (from 0.95 ± 0.03 at resting ventilation to 0.47 ± 0.03 at 30 L/min) when oxygen

flow was set to 8 L/min ($p<0.001$). HiOx80 provided an $FIO_2 > 0.4$ at minute ventilations of ≤ 18 L/min with oxygen flow of only 4 L/min ($p<0.001$). When oxygen flow was further reduced to 2 L/min an $FIO_2 > 0.4$ was achieved at minute ventilations of <10 L/min ($p<0.05$). FIO_2 delivered by Hudson and HiOx80 masks decreased significantly ($p<0.05$) with increasing minute ventilations, whereas FIO_2 with the Venturi mask did not change significantly from 0.4 ($P>0.05$).We conclude that HiOx80 should be used preferentially to provide high $FIO_{2,}$ as well as for efficient delivery of O_2 when supplies are limited.

137. THE TIEN SHAN MOUNTAINS (KAZAKHSTAN): THE NEW FRONTIER OF ALTITUDE RESEARCH

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Suitable facilities must be readily accessible, inexpensive, and have adequate infrastructure to support instruments used for high altitude research. Researchers tend to frequent the same, but limited facilities in the Alps, Himalayas and Andes. We engaged in a pilot project to explore the feasibility of adding the Tien Shen Mountains in Kazakhstan to the list of suitable venues. A group of 4 Canadian altitude researchers joined The Kazakhstan Centre for Disaster Medicine and Republican Search & Rescue Team to carry out three studies. The local teams arranged for local transportation, shelter, electrical access, communications, backup medical and rescue support, and necessary provisions for the destination altitudes. They stayed on to provide continuous logistic support including volunteering as study subjects. Among the resources we used were all-terrain vehicles, horses, electrical generators, large tents, heaters, portable blood gas and biochemical laboratory. Helicopters are available but we did not require them. Accessible altitudes include 2200 m (Chimbulak) and 4000 m (Amangeldi). Access to altitudes up to 7000 m requires advanced climbing skills. Local search and rescue personnel are prepared to pre-install safety lines along the rout to camp and provide climbing support. Heavy equipment can be delivered by helicopter. The entire region is found within close vicinity of major cities with access to modern medical, communication and transportation facilities. The high altitude region is most accessible from mid-July to mid-September. Cost is on the basis of cost recovery (fuel, personnel). Cost is further defrayed in collaborative studies by each party bearing expenses.Contact information will be provided.

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138. ALTERATIONS IN COGNITIVE FUNCTION WITH O2 SUPPLEMENTATION AT MODERATE ALTITUDE

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It is not clear to what extent cognitive function is altered by hypoxia or oxygen supplementation at moderate altitude. We performed a randomized double blind study of physi-

ological and cognitive response at 800 m and 3200 m before and after administration of supplemental O_2 via HiOx80 at 1 L/min in 8 healthy male volunteers. The ANAM battery of tests was used to assess reaction time, mathematical processing, procedural memory and code substitution. In comparison to 800 m, at 3200 m, oxygen saturation decreased (to 90±1% from 97±0 %) and heart rate increased (to 107±4 bpm from 80±4 bpm). Supplemental O_2 at 3200 m significantly increased arterial oxygen saturation (90±1 vs. 97±0 %) and decreased heart rate (94±5 vs. 107±4 bpm), but had no effect on these variables at 800 m. No changes in cognitive function were identified under any condition. The ANAM tests did not detect cognitive deterioration with acute exposure to low-moderate altitude, possibly because of inadequate hypoxic stimulus. Low flow O_2 supplementation at 3200 m ameliorates select physiological effects of altitude.

139. DOES CEREBRAL VASCULAR REACTIVITY ACCLIMATIZE

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At altitude, hypoxia and hypocapnia result in changes in cerebral blood flow (CBF), as defined by the subject's underlying cerebral vascular reactivity (CVR) curve. It is important to characterize the exact nature of this response in order to understand its role in maintaining cerebral oxygen delivery, and its possible role in the pathogenesis of HACE. We hypothesized that, similar to ventilatory chemoreflexes, CVR may show acclimatization. One female and five male healthy volunteers were studied at sea level and after 5 days at 3480 m. Cerebral blood flow velocity (CBFv) was measured using transcranial doppler ultrasound. CVR was assessed as the response of CBFv over a continuous range of $PetCO_2$ at two iso-oxic values of $PetO_2$, using modified Read rebreathing tests with iso-oxia maintained at $PetO_2$ of 50 mmHg or 150 mmHg. Each subject performed one hypoxic and one hyperoxic rebreathing test at sea level prior to ascent (SL), and after 5 days at altitude (Alt). T-test was used to compare slopes of linear fits to CBFv versus $PetCO_2$ data. In three subjects, CBFv increased continuously with rising PCO_2, however surprisingly, in the other three subjects, CBFv plateaued before maximally tolerated PCO_2 was reached – this was not an artifact from signal cutoff. A trend towards increased CVR at altitude was seen but did not reach statistical significance due to intersubject variability. Resting PCO_2 (mmHg) - SL: 39.9 +/- 1.5, Alt: 31.6 +/- 1.7 ($p<0.05$); Resting CBF (cm/s) - SL: 47 +/- 2, Alt: 50 +/- 3; CVR (cm/s/mmHg) - SL: 1.33 +/- 0.12, Alt: 1.43 +/- 0.24.1. Further study is needed, however these data imply that cerebral vascular reactivity may acclimatize by increasing in sensitivity. This may help to preserve CBFv and cerebral oxygen delivery in the face of falling $PetCO_2$ due to ventilatory acclimatization. It appears that in some subjects CBFv can reach a maximum at relatively low PCO_2.

140. ISOCAPNIC AND POIKILOCAPNIC HYPOXIA VENTILATORY RESPONSE CONSENSUS METHODS IN HUMANS

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At the 13th Hypoxia Symposia (2003), consensus methods were proposed for measuring hypoxic ventilatory responses (HVR) during 20 min of both isocapnic (IH) and poikilocapnic (PH) hypoxia, and for better predicting success in altitude acclimatization. During IH, HVR at 5 min quantifies peripheral chemoreceptor stimulation to hypoxia per se while HVR at 20 min measures hypoxic ventilatory decline (HVD). During PH, HVR at 5 min tests the interaction of peripheral chemosensitivity with the concomitant hypocapnic alkalosis while the value at 20 min includes HVD, as occurs at altitude. We examined IH and PH HVR in 7 normal adults, age 26±3yrs, during two randomly assigned 20 min protocols. During IH, $PETCO_2$ was held at 38±1.0 Torr (1 Torr above resting values) while $PETO_2$, was reduced stepwise using dynamic end-tidal gas forcing to 45 Torr. During PH, $PETO_2$ was reduced stepwise to 45 Torr with zero inspired CO_2. Tests were separated by 1 hour. During IH, iHVR calculated as $\Delta VE/\Delta SpO_2$ (L/min/%) was 2.37±0.61 and 0.78±0.27 at 5 and 20 min, respectively ($P<0.05$). HVD was thus 43.7±5.4 %. During PH, $PETCO_2$ fell 5 and 6 Torr at 5 and 20 min, respectively, although VE was not measurably increased. pHVR reported as $\Delta PETCO_2/\Delta SpO_2$ (mmHg/%) was 0.33±0.05 and 0.28±0.08 at 5 and 20 min, respectively during PH. During IH, SpO_2 was 81% while during PH it fell from 80.2±1.6% (5 min) to 74.8±1.8% (20 min). During IH, iHVR was correlated to both pHVR ($r = 0.63$, $P<0.05$) and change in $PETCO_2$ ($r = -0.41$, $P<0.05$) during PH. Further research is required to assess whether these combined indices of HVR are useful for predicting acclimatization success to altitude.

Acknowledgements: This study was approved by the local Ethics Board and supported by AHFMR, HSFA, CIHR.

141. DO THE CAROTID CHEMORECEPTORS MODULATE REGIONAL BLOOD FLOW DISTRIBUTION AND VASCULAR CONDUCTANCE DURING EXERCISE

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Stimulation of the carotid chemoreceptors (CC) causes increased sympathetic vasoconstrictor outflow to skeletal muscle. We examined the effect of inhibiting/stimulating CC on cardiac output (QT), hind-limb flow (QL), total (GT) and hindlimb (GL) conductance and blood pressure (BP) during exercise (2.5 mph, 5% grade) in the dog.Bolus injections (<0.5 ml) of Dopamine (10 ug/kg) or NaCN (2 ug/kg) were administered via a chronically implanted carotid arterial catheter. Dopamine has been previously shown to have a bi-phasic response on carotid sinus nerve activity, with a 1-2 sec excitatory period followed by a prolonged (5-10 sec) inhibitory period, while NaCN injections cause only excitation (Bisgard et al., 1979). During exercise, injections of Dopamine elicited a bi-phasic response; first, a short (2 sec) reduction in GT (7%) and GL (15%) was observed. This was followed by a

pronounced vasodilation (5-10 sec) as indicated by increases in GT (24%), GL (31%), QT (7%), and QL (14%), and a 10% reduction in BP. NaCN caused a similar initial transient decrease in both GT (-7%) and GL (-11%), however there was no evidence of a reflex vasodilation as little change in GT (5%), GL (5%), and BP (-2%) was observed during the ensuing period. Isovolumetric control injections of saline elicited no cardiovascular response. These preliminary results indicate that modulation of the carotid chemoreceptors can affect the distribution of blood flow during exercise, likely by influencing sympathetic nervous activity.

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142. ERYTHROPOIETIN AND ACCLIMATISATION: MORE THAN JUST ERYTHROPOIESIS

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Erythropoietion has a long appreciated role in adaption to high altitude in relation to haemopoiesis. More recently erythropoietin has been shown to have cytoprotective properties, protecting the brain, heart and kidneys from ischaemic injury. We hypothesised that erythropoietin may play a role in the early phase of acclimatisation independent of its erythropoietic activity. On a recent expedition to Bolivia (APEX2) we examined the relationship between serum erythropoietin and Acute Mountain Sickness (AMS). 41 healthy lowlanders (25 male, median age 21 range 18 to 31) flew to La Paz, Bolivia (3650m) spending 4 or 5 days before ascending in 90 minutes to Chacaltaya (5,200m). We measured serum erythropoietin in venous blood at sea level (SL1), within 6 hours of arrival at 5,200m (CH1), day 3 (CH3) day 6 (CH6), and again at sea level 2 months later (SL2). AMS was scored using the Lake Louise Scoring system (LLS).There was a 78% incidence of AMS in the first 3 days at 5200m (LLS>3). Maximum AMS scores in the first 3 days following ascent to 5200m were used to compare serum erythropoietin responses of subjects with little or no AMS (‘AMS-ve’, LLS<3, n=9) to those with moderate to severe AMS (‘AMS+ve’, LLS>4, n=32). Mean erythropoietin levels (IU/L±SEM) were as follows: AMS-ve 10.3±1.6 (SL1), 24.1±2.4 (CH1), 124.8±29.0 (CH3), 79.3±22.4 (CH6), 11.2±1.5 (SL2); AMS+ve 10.3±0.6 (SL1), 21.0±1.6 (CH1), 57.0±4.9 (CH3), 54.1±9.4 (CH6), 10.2±0.7 (SL2). Mean serum erythropoietin 48 hours after arrival at 5200m (CH3) was markedly higher in the AMS-ve group (124.8±29.0 vs. 57.0±4.9, $p<0.007$, two-tailed Mann-Whitney test).These results suggest that a blunted erythropoietin response to acute altitude exposure may be associated with AMS. The potential for erythropoietin to protect the brain and other organs from hypoxic injury in altitude acclimatisation deserves further investigation.

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143. ORTHOSTATIC HYPOTENSION IS COMMON AT ALTITUDE AND ASSOCIATED WITH PRE-SYNCOPE

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Orthostatic hypotension, assessed by tilt table, is greater at altitude. The blood pressure (BP) response on standing upright is of interest because of the increased incidence of syncope at altitude and the increased prevalence of dizziness, a symptom of acute mountain sickness.We investigated orthostatic hypotension at altitude and its association with symptoms of pre-syncope on standing up from a supine position, in healthy volunteers. 36 lowlanders (22 males, 14 females) flew into La Paz, Bolivia (3650m) and after 4-5 days acclimatization ascended over 90 minutes to the Chacaltaya laboratory (5200m) by off-road vehicle. BP was measured on five occasions, three times at 5200m, and twice at sea-level, before and after the expedition. BP was measured with a mercury sphygmomanometer; a supine measurement and an erect measurement, within 15 seconds of standing, were recorded. The mean fall in BP for males at the first sea-level sample day was 11.9 mmHg, compared to 20.7 mmHg (paired t-test, $p = 0.0224$, $n = 19$), 27.8 mmHg ($p < 0.0001$, $n = 17$) and 25.0 mmHg ($p = 0.002$, $n = 17$) on the first, third and seventh days at altitude respectively. For females the fall in systolic BP was greater at altitude, but the difference was not significant. Symptoms (feeling faint on standing) were more prevalent on all altitude sample days, although only significant ($p<0.01$) on the first day (81%), in comparison with sea-level sample days (45% and 50%). Symptoms were associated with a greater fall in systolic BP at altitude, although the differences were not significant. Orthostatic hypotension is more severe at altitude, particularly amongst men, and is likely to be associated with symptoms of pre-syncope. Dehydration or changes in the regulation of venomotor tone are the likely aetiology.

Acknowledgements: APEX2 Volunteers

144. EFFECT OF INTERMITTENT NORMOBARIC HYPOXIA ON PLASMA GLUTAMINE CONCENTRATION IN TRAINED MEN

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The purpose of the present study was to investigate the effect of intermittent normobaric hypoxia on plasma glutamine, which is one of the energy substrates for immune cells, and on immune parameter in trained men.Eight male triathletes and five male cyclists volunteered for this study. They were randomly divided into a hypoxic (H) group (n=7) or control (C) group (n=6). H group stayed in a normobaric hypoxic room (15.4% O_2) for 10 hours a day (22:00-8:00) over 10 days whereas C group stayed in normobaric normoxic condition. Both groups maintained their usual training routine at sea level during the experimental pe-

riod. To measure plasma glutamine concentration and parameters of CBC/differential and reticulocyte, blood samples were drown from antecubital vein at baseline before the experiment, and in the morning of day 1, 3, 6 and 10. Maximal oxygen uptake was also evaluated before and after the experimental period. The plasma glutamine concentration in H group significantly ($P<0.001$) decreased by 23% within one day and then gradually recovered to basal level by day 6, but it once again significantly dropped on day 10. Lymphocyte count in H group was significantly ($P<0.01$) lower on day 1 and 6 compared to baseline. There was no change in maximal oxygen uptake over the period. The results suggest that a significant decrease in plasma glutamine associated with an intermittent normobaric hypoxia may have a negative influence on immune function in trained men.

145. THE EFFECT OF SILDENAFIL ON CEREBRAL OXYGENATION AT ALTITUDE

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Sildenafil has been shown to reduced pulmonary hypertension and improve arterial oxygenation at altitude. We studied the effect of sildenafil on cerebral perfusion and oxygenation at altitude. Previously, we have shown middle cerebral artery velocity (MCAV) tends to fall and cerebral oxygenation (rSO_2) rises one hour after sildenafil when given three days after arrival at altitude. We have extended these observations to 1 and 2 hours after sildenafil and on one and three days after arrival at altitude (3480m) in a larger group of subjects (n=10). Results are given as mean (standard error). Results Day 1: Heart rate increased, blood pressure decreased and arterial oxygen saturation increased at both 1 and 2 hours after sildenafil. MCAV was unchanged but rSO_2 increased from 59.35% (1.3) to 62.7 (0.8) ($p<0.05$) at 1hour and to 65.3 (0.9) at 2 hours. End tidal CO_2 was unchanged. Results Day 3: Heart rate and blood pressure changes were similar as on day 1 but arterial oxygen saturation was unchanged at 1 and 2 hours. MCAV was reduced from 65.3 cm.sec-1 (1.8) to 61.3 (1.5) ($p<0.01$) and to 60.9 (1.7) ($p<0.0001$) at 1 and 2 hours respectively. rSO_2 increased from 61.7 % (0.9) to 65.0 (1.0) ($p<0.0001$) at 1hour and to 64.0 (0.9) ($p<0.0001$) at 2 hours. End tidal CO_2 decreased from 4.088 kPa before sildenafil to 4.010 kPa at 2 hours ($p<0.01$). Sildenalfil increases cerebral oxygenation at altitude. On day 1 rSO_2 increased still further at 2 hours without any change in MCAV. On day 3 no further rise in rSO_2 was found after 1hr and the rise in rSO_2 was accompanied by a fall in MCAV. The mechanisms whereby sildenafil increases rSO_2 may be different on acute exposure to altitude and after acclimatisation.

146. HYPOXIA INDUCIBLE FACTOR-1A REGULATES IN VIVO T CELL RESPONSES

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A basic HLH-PAS transcription factor, hypoxia inducible factor-1a (HIF-1α) facilitates the adaptation of cells to oxygen deprivation, by regulating the genes that are involved in glucose uptake, angiogenesis, erythropoiesis, and cell survival. The roles of HIF-1α in immune system have been identified in myeloid cells and in developing B cells. To investigate the role of HIF-1α in T cells, HIF-1α -floxed mice were crossed with proximal lck promoter-driven Cre-transgenic mice. We found that HIF-1α -deficient T cells were normally generated in the thymus and their distribution in the spleen and lymph nodes was unimpaired. In the in vitro culture conditions, HIF-1α -deficient T cells exhibited undiminished IL-2/IL-2R production and proliferation upon stimulation with either anti-CD3 antibody or Con A. However, any T cell-mediated responses measured in vivo by Con A-induced IL-2R expression, SEB-induced increase and decrease of Vb8+ cells, and SRBC-induced antibody production were severely diminished in T cell-specific HIF-1α -deficient mice. Con A treatment in T cell-specific HIF-1α -deficient mice caused less severe hepatitis.These results indicate that HIF-1α in T cells plays a crucial role in the in vivo immune responses, suggesting a pivotal role of hypoxic responses in intravital T cell-mediated immunity.

147. WEIGHT LOSS AT HIGH ALTITUDE: A BENEFICIAL EFFECT FOR OBESE SUBJECTS

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Loss of weight is frequently observed at high altitude and is believed a harmful effect for people who live at high altitude. However, the beneficial effect of weight reduction at high altitude has currently not been determined. The present study was performed to evaluate high-altitude weight loss as a nonpharmcotherapic effect for obese subjects when they are exposed to high altitude. A 120 subjects (age 34.5± 4.6 yr) who worked in the construction of Golmud-Lhasa Railway (4600m), which is the highest railway in the world, participated in the study. 85 subjects came from sea level (sea-level group) and 35 came from moderate altitude (2200m; moderate-altitude group). Body weight and body mass index (BMI) were measured at Golmud (baseline; 2800m) and after one month after they ascended to an altitude of 4600m. Among the sea-level group, the body weight of 20 subjects at 4600m was measured each morning for 33days continuous before they went to work. The average weight loss for the see-level group (n=85) was 10.2±3.8%, with the highest one of 29%, and 2.3%± 0.6% for the moderate-altitude group. The degree of weight loss (Δ weight loss) in the sea level group was significantly correlated with their baseline BMI ($r=0.678$; $p<0.001$) but this was not true in the moderate-altitude group ($r=0.009$). In the 20 subjects who had continuous measurements of body weight significant weight loss was observed within 20days of ascent but was a stable thereafter. The main findings of the present study are that 1) high altitude exposure markedly reduces body weight in sea level subjects but not in moderate -altitude subjects; 2) the degree of weight loss is closely related to initial

BM in the sea-level group; 3) a sufficient weight loss occurs within three weeks when people from near sea-level ascend to high altitude. Conclusion: These results suggest that weight loss at high altitude may be a safe, but previously unrecognized nonpharmcologic strategy for the treatment of obesity.

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148. INDEPENDENT AND COMBINED EFFECTS OF HYPOXIA AND ELEVATED ESOPHAGEAL TEMPERATURE ON THE PATTERN OF HUMAN EXERCISE VENTILATION

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The independent and combined effects of hypoxia and elevated esophageal temperature (Tes) were investigated for their effects on the pattern of exercise ventilation (VE). Eleven college-aged, healthy males volunteered for the study. In either a 'hyperthermic' Tes or a 'normothermic' Tes exercise session, participants were immersed to their shoulders and pedalled on a head-out, underwater cycle ergometer at a steady-state oxygen consumption (O2) of 0.87 (SD 0.07) L•min-1. A bath temperature of 31.5ºC (SD 1.4) gave a steady state Tes of ~37.1ºC for the normothermic exercise and a bath temperature of 38.2ºC (SD 0.1) gave a steady state Tes of ~38.6ºC for hyperthermic exercise. After a 30-min rest in air, participants were immersed and performed a 20-min warm-up followed by a 30-min steady-state cycling period when they inhaled air for 10 min, then hypoxic gas (12% O2 and 88% N2) for the next 10 min and then air again for 10 min. End-tidal CO2 (PETCO2) was maintained at an isocapnic level of 5.19 kPa (SD 0.71) during exercise. Results showed a main effect of core temperature ($P = 0.007$) was evident for breathing frequency (f), with an increased f in hyperthermic relative to the normothermic exercise. Significant decreases in inspiratory time (TI; $P = 0.035$) and expiratory time (TE; $P = 0.014$) were evident and these decreases were independent of any changes in tidal volume (VT). Inspiratory flow (VT•TI-1) was significantly increased in hyperthermic relative to normothermic ($P = 0.003$) exercise, an increase that was pronounced ($P = 0.013$) during hyperthermic hypoxia. In conclusion, during low intensity, steady-state exercise, a hyperthermic relative to a normothermic Tes caused a significant increase in VE due to an increase in f on account of decreases of TI and TE. This gave evidence of a thermally-induced tachypnea with increases of inspiratory flow that were accentuated in hyperthermic, hypoxic exercise.

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149. GINKGO BILOBA PROPHYLAXIS DOES NOT PREVENT ACUTE MOUNTAIN SICKNESS

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Acetazolamide and ginkgo biloba have both been recommended for AMS prophylaxis; however, there is conflicting evidence of efficacy for ginkgo. We performed a prospective randomized, placebo-controlled trial of acetazolamide versus ginkgo biloba for AMS prophylaxis.We randomized unacclimatized adults to receive acetazolamide, ginkgo biloba, or placebo in a double-blinded fashion and took them to elevation (3800m) for 24 hours. For 5 days prior to ascent, subjects took identically appearing capsules twice daily containing either placebo, or Ginkgo biloba 120 mg. Subjects randomized to the acetazolamide group received placebo for 5 days, then were switched to acetazolamide 250 mg twice daily one day prior to ascent . We graded AMS symptoms using the Lake Louise Score (LLS) and compared the incidence of AMS (defined as LLS >3 and headache). Fifty seven subjects completed the trial (20 acetazolamide, 17 ginkgo biloba, 20 placebo). LLS scores were significantly different between groups; the median acetazolamide score was significantly lower than placebo (p=0.0124, effect size 2, 95%CI 0, 3) unlike ginkgo biloba (p=0.889, effect size 0, 95%CI -2, 2). AMS occurred less frequently in acetazolamide subjects compared to placebo (effect size 30%, 95%CI 61%, -15%), and was similar between ginkgo biloba subjects compared to placebo (effect size -5%, 95%CI -37%, 28%). In this small study, prophylactic acetazolamide decreased the severity and incidence of AMS. We found no evidence of similar efficacy for ginkgo biloba.

150. A ROLE FOR ACTIGRAPHY AS AN ADJUNCT TO SLEEP MONITORING AT HIGH ALTITUDE

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Sleep-disruption is almost universal at high altitude, however many sleep systems are cumbersome to use in a remote environment. Actigraphs, or movement sensors, may be used as an indicator of sleep or wakefulness. Their simplicity is attractive for altitude use, but they have not to our knowledge previously been evaluated in this environment. Objectives: (1) Evaluation of nocturnal-movement with ascent (2) Determination of the relationship with subjective sleep quality. 15 volunteers underwent a graduated 17-day ascent from Tumlingtar (350m) to Chamlang base-camp (5000m). They wore an Actigraph (Cambridge Neurotechnology, UK) on the non-dominant wrist. At basecamp data was downloaded and the Fragmentation Index (FI) for each night determined. VAS was recorded each morning, using a 100 mm rule (0= 'best night's sleep ever': 100= 'worst night's sleep ever'). Examining data for new ascents only: FI increased significantly from 22.2 +/- 10.0/hour at 410m,

through sequential altitudes to 37.1 +/- 17.9/hour at 5000m (p=0.001, ANOVA). There was no significant change in subjective sleep quality (VAS score 64.6 +/- 22.0 at 410m and 57.5 +/- 22.3mm at 5000m, p=0.25). There was a significant relationship between FI vs altitude (r2=0.40; p=0.006, Pearson's correlation coefficient) and FI vs VAS (r2=0.33; p=0.019), and a trend towards a relationship between VAS and altitude (r2=0.22; p=0.058). We have demonstrated that nocturnal movement increases sequentially with ascent. Using VAS as the primary outcome, subjective sleep quality did not significantly deteriorate with altitude, and this may be attributable to our slow ascent profile. Despite this, we demonstrated a significant relationship between nocturnal movement and subjective sleep. We have also demonstrated this simple device to be robust and reliable in the remote high altitude environment, despite the restriction of a sleeping bag. It may make a useful adjunct to sleep monitoring at altitude.

151. ELEVATED CEREBRAL BLOOD VOLUME DURING HYPOBARIC HYPOXIA PROTECTS AGAINST THE DEVELOPMENT OF AMS

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Acute mountain sickness (AMS) is a common ailment among individuals who travel too high too fast. The etiology of AMS remains poorly understood, in part due to the difficulty in obtaining non-invasive measurements of many cerebral parameters. Elevated cerebral blood volume (CBV) has been implicated by several authors as a contributor to the development of AMS. Traditionally, measurement of CBV has required creating a change in blood arterial oxygen saturation levels and comparing the change in concentration of oxygenated hemoglobin monitored by near infrared spectroscopy (NIRS), to that occurring in a peripheral pulse oximeter. In most reports this technique, known as the oxygen method, has been limited by poor reproducibility. Alternatively, NIRS can be used to monitor changes in total hemoglobin, as a surrogate for changes in CBV, but is limited by an inability to absolutely quantitate CBV. To apply this technique to longer duration studies we developed a unique NIRS optode holder and placement technique that allowed us to reproducibly replace the optodes (coefficient of variation = 1.3%) and sample the same "walnut" of cerebral tissue over time. We used the two different techniques to measure changes in CBV in altitude naive individuals exposed to hypobaric hypoxia (Pb =426 mmHg) for nine hours. Using the total hemoglobin method, we found that CBV increased in all subjects. Interestingly, these changes were largest in the AMS negative individuals ($p<.01$ at 9hrs). The magnitude of this difference correlates to a change in total CBV of 0.45 ml/100g. A similar although non-significant, trend was seen using the quantitative CBV technique. We hypothesize that individuals who will develop AMS may not be able to further increase their CBV, and subsequently increase oxygen delivery, secondary to poor craniospinal capacitance.

152. INHIBITION OF EXTRACELLULAR CARBONIC ANHYDRASE DOES NOT PREVENT HYPOXIC PULMONARY VASOCONSTRICTION

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Objective: Combined inhibition of both intra- and extracellular carbonic anhydrase (CA) by acetazolamide has been shown to prevent hypoxic pulmonary vasoconstriction (HPV). This study investigates, whether this effect is primarily due to extracellular CA inhibition, using benzolamide (BZ) - a powerful, hydrophilic, nonpermeant CA inhibitor - to inhibit extracellular luminal membrane bound CA isoenzyme IV. Methods: Six Beagle dogs were studied twice in randomized order. Protocol 1: Controls; Protocol 2: benzolamide IV (BZ) (2mg/kg bolus, followed by 2mg/kg/h continuously). During all experiments the conscious dogs were breathing spontaneously through a face mask: first hour normoxia (FiO_2=0.21), followed by 2 hours of hypoxia (FiO_2=0.1 in Controls and 0.09-0.08 in the BZ protocol, to match the PaO_2 observed during hypoxia in Controls). Mean pulmonary artery pressure (MPAP) and pulmonary vascular resistance (PVR) were recorded. Arterial blood gases were measured during normoxia and after 2 hours of hypoxia. Data are means±SEM; $p<0.05$ (GLM-ANOVA). Results: PaO_2 and $PaCO_2$ during hypoxia were 35.8±2.4mmHg and 29.1±0.9 mmHg in Controls and 38.3±1.2mmHg and 27.8±0.7mmHg with BZ. MPAP increased from 13.3±1 (normoxia) to 20.7±1.3mmHg (hypoxia), and PVR increased from 324±33 to 528±37dyn·s-1 ·cm-5 in Controls. These values were comparable in the BZ protocol (MPAP 11.6±0.7 vs 18.6+1.1mmHg; PVR 251±14 vs 508±46dyn·s-1·cm-5). Arterial pH increased from 7.39±0.01 (normoxia) to 7.46±0.01 (hypoxia) in Controls while base excess (-2±0.6mmol/l) did not change. With BZ, arterial pH was 7.36±0.01 (normoxia) and 7.41±0.01 (hypoxia), while base excess decreased from -4.25±0.2 to -6.2±0.4mmol/l. Conclusions: BZ could not prevent HPV in conscious, spontaneously breathing dogs, suggesting that prevention of HPV by CA inhibition is not primarily related to extracellular vascular endothelial CA (IV) inhibition and changes of systemic pH.

CORRECTIONS

The following abstracts are presented here with corrections from the original as printed in the Hypoxia Abstract issue of High Altitude Medicine and Biology. The corrections will appear in the summer 2005 edition of High Altuitude Medicine and Biology.

The author line was mis-printed on the following abstracts.

6. EFFECTS OF SHORT-TERM NORMOBARIC HYPOXIA ON ANAEROBIC AND AEROBIC PERFORMANCE IN HIGHLY TRAINED ATHLETES

Fabien A. Basset[1], Denis R. Joanisse[2], François Billaut[3], Frédéric Boivin[2], Jean Doré[2], Josée St-Onge[2], Richard Chouinard[2], Guy Falgairette[3], Denis Richard[2], and Marcel R. Boulay[2].
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82. INFLUENCE OF HYPOXIA ON EXHALED NITRIC OXIDE, PULMONARY FUNCTION, AND PULMONARY VASCULAR PRESSURES IN HEALTHY HUMANS

Eric Snyder, Ken Beck, Robert Frantz, Bruce Johnson.
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92. REGULAR SILDENAFIL DOES NOT INHIBIT ALTITUDE-INDUCED PULMONARY HYPERTENSION; A RANDOMISED DOUBLE BLIND PLACEBO-CONTROLLED TRIAL

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The body of the following abstract was not properly reproduced in HAMB:

14. PULMONARY FUNCTION AND NOCTURNAL VENTILATION IN MOUNTAINEERS DEVELOPING HAPE

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Introduction: To investigate changes in lung function, breathing patterns, and oxygenation in montaineers developing HAPE. Methods: We studied 18 mountaineers in Zurich (490m) and after ascent to Capanna Margherita (4559m), within <24 hours. Eight mountaineers developed HAPE, 10 remained well (controls). Lung function, nocturnal breathing pattern by calibrated inductive plethysmography, and oxygen saturation were measured in Zurich (490m) and at 4559m over 3 consecutive days or until HAPE was clinically and radiographically diagnosed. Results: HAPE developed in 8 subjects within the 3 days at 4559m. Their FVC progressively decreased from a mean ±SD of 108±17 %pred at 490m to 87±15 %pred at 4559m (P<0.05, last measurement before overt HAPE). FEV1 fell in proportion to FVC. Closing volume increased from 0.33±0.06 to 0.51±0.05 L (P<0.05). Maximal inspiratory pressure remained unchanged. Diffusing capacity decreased from 102±11 to 91±8 %pred (P<0.05). Mean oxygen saturation in the night before HAPE occurred was 60±8%, the number of periodic breathing cycles was 97±45 h-1, minute ventilation was 8.6±3.1 L/min, breath rate was 27±5 min-1, mean inspiratory flow was 0.33±0.01 Lmin-1. In controls, FVC and FEV1 decreased to a similar degree as in subjects developing HAPE, but changes in closing volume (from 0.40±0.10 L at 490m to 0.41±0.07 L at 4559m, P=NS) and diffusing capacity (from 103±14 %pred at 490m to 102±16 %pred at 4559m, P=NS) were less pronounced. Nocturnal oxygen saturation in controls was higher (73±3 %), periodic breathing cycles (48±44 h-1), minute ventilation (5.1±1.8 Lmin-1), breath rate (20±3 min-1), and mean inspiratory flow (0.19±0.06 Lsec-1) were lower than in subjects developing HAPE (P< 0.05, all comparisons). Conclusion: Overt HAPE is preceeded by changes in pulmonary function, gas exchange and breathing pattern suggesting insterstitial fluid accumulation but not a reduced respiratory center drive. Measurement of pulmonary function and nocturnal breathing pattern might assist in early detection of HAPE.

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