ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 618

HYPOXIA AND THE CIRCULATION

Edited by
Robert C. Roach
Peter D. Wagner
and
Peter H. Hackett

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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 22 countries joined together in February 2007 for four days of intense scientific discourse in the dramatic mountain setting of Lake Louise, Canada.

The 15th 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 fifth meeting as the organizers, we were especially pleased that the Hypoxia Meetings continue to prosper. We remain always thankful for the kind and wise guidance of Charlie Houston, the originator of the Hypoxia meetings.

We strive to maintain a 30-year tradition of presenting a stimulating blend of clinical and basic science papers focused on hypoxia.Topics for 2007 included the risk of heart disease at high altitude, and the regulation of stroke volume and coronary blood flow. Also covered were metabolic, cognitive and vascular consequences of intermittent hypoxia, vascular remodeling in different vascular beds, lung fluid movement in hypoxia, new work on globins, including neuroglobin, myoglobin and genetic regulation of hemoglobin mass. Hypoxic responses in insects and the hypoxic skeletal muscle rounded out the regular sessions. We also had tributes to the 2007 Hypoxia Honoree, Professor James Milledge, and a special tribute to our late friend, Dr. Carlos "Choclo" Monge Cassinelli.

The abstracts from the 2007 meeting were published in High Altitude Medicine & Biology Dec 2006, Vol. 7, No. 4: 319-350. Late abstracts are presented in the last chapter of 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 2007 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, the John Sutton Fund from McMaster University, and our International Advisory Committee. At the meeting we were greatly helped by Barbara Lommen, Paige Sheen, Kelly Brown, 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 2009 at the Chateau Lake Louise, Lake Louise, Alberta, Canada for the 16th International Hypoxia Symposium.

Robert C. Roach, Peter D. Wagner, Peter H. Hackett, Editors, May 2007
(www.hypoxia.net)

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Chapter 1

RISK OF CARDIOVASCULAR EVENTS DURING MOUNTAIN ACTIVITIES

Martin Burtscher
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Abstract: Sudden cardiac death (SCD) is the major cause of fatalities in males over 34 years of age during hiking or downhill skiing in the mountains. The main goal of the present study was the identification of risk factors and triggers associated with SCDs during these mountain activities. Besides recording individual circumstances associated with SCD, a case-control study was performed comparing the risk factor profiles of 247 males over the age of 34 who suffered SCD during mountain hiking or downhill skiing with those of 741 matched controls. The SCD risk was greatest on the first day at altitude but altitude per se and the duration of activity did not appear to markedly modify this risk. In contrast, the longer the time from the last food and fluid intake during hiking, the higher was the SCD risk. Early cardio-pulmonary resuscitation was started in 33 % of skiers and in 14 % of hikers after occurrence of unconsciousness. Hikers who died suddenly during mountain hiking were much more likely to have had a prior myocardial infarction (MI) (17 % vs. 0.9 %), known coronary artery disease (CAD) without prior MI (17 % vs. 4 %), diabetes (6 % vs. 1 %), hypercholesterolemia (54 % vs. 20 %), and were also less engaged in regular mountaineering activities (31 % vs. 58 %) compared with hikers from the control group (all $P < 0.001$). Skiers who suffered SCD had much more frequently a prior MI (41 % vs. 1.5 %), hypertension (50 % vs. 17 %), known CAD without prior MI (9 % vs. 3 %), and were less engaged in regular strenuous exercise (4 % vs. 15 %) when compared to controls (all $P < 0.05$). These findings enable identification of skiers and hikers at increased SCD-risk and recommendation of preventive measures, e.g. pharmacological interventions and adaptation to specific mountain activities. They also underline the need for intensified cardio-pulmonary resuscitation training for all mountaineers.

Key Words: sudden cardiac death, downhill skiing, mountain hiking, exercise, regular physical activity

INTRODUCTION

The Alps comprise the largest and most popular sports region in Europe. Austria accounts for almost one third of the 180,000 km^2 of mountainous area. In Austria alone, each year more than 10 million persons from practically every country in the world are

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involved in one of the many mountain activities (downhill skiing, mountain hiking, ski-touring, rock climbing, ice-climbing, snow boarding, mountain biking, paragliding, etc.). About 85 percent of these people are downhill skiers and/or mountain hikers (4). Whereas mountain sports activities can undoubtedly contribute to fitness and longevity, they are also combined with a relatively high risk of death (2, 6). In Austria there are about 300 fatalities during mountain sports annually. About 30 % of these deaths are non-traumatic deaths, mostly sudden cardiac deaths (SCDs) (4). Based on accumulating reports on fatal cardiac events in hikers and skiers during the peak vacation periods, the impression arises that downhill skiing, like mountain hiking in summer, is associated with a particularly high risk of SCD. Numerous studies have estimated the frequency of SCDs to the general public and during vigorous exercise (10, 15, 24, 25, 29, 35). However, relatively little data are available on SCDs which occur during hiking or downhill skiing (7, 8).

For this reason, the main objectives of the present paper are the estimation of the SCD-risk during downhill skiing and mountain hiking and the identification of main risk groups and risk factors associated with SCD.

METHODS

The recording of fatalities during mountain sports activities in Austria, the estimation of the respective population at risk, and the case-control analyses for the determination of risk factors have recently been described elsewhere (3, 4) and will only be presented here briefly.

Fatalities during mountain sports activities and diagnosis of deaths

All fatalities during mountain sports activities in Austria within a nine year period were recorded by qualified alpinists with para-medical training. Data encompassed characteristics of the mountaineers (age, sex, nationality, the type of mountain sport practiced, etc.), the circumstances of the fatality, the doctor's diagnosis and further details like terrain, altitude, and weather conditions. The diagnosis of "sudden cardiac death" has been made by the emergency physician, by the doctor in the hospital and sometimes additionally on the basis of the results of an autopsy which was performed in about 10% of all cases.

Sudden cardiac death is defined as unexpected, non-traumatic death in persons with or without pre-existing disease who die within 1 hour of the onset of symptoms with exclusion of CVA and PE (9, 20).

The population at risk

The total number of hikers and skiers is based on a representative Austrian-wide survey which has been carried out to determine the number of persons involved in individual mountain sports activities, differentiation according to age and sex, and the frequency of involvement in (alpine) sports (26), on data collected among a representa-

tive sample of hikers and skiers in Austria (13), and on microcensus (1, 16).

The case-control-study

Cases: All deaths which occurred during mountain hiking and downhill skiing during a nine year period in Austria were recorded. Males > 34 years of age who suffered SCD during mountain hiking or downhill skiing and who were residents of Austria or Germany were eligible for inclusion in the study. Rare cases in which cardiovascular processes such as intracerebral hemorrhage, pulmonary embolism and dissecting aortic aneurysm were demonstrated were excluded. Out of all recorded cases (n = 518) with SCD 405 fulfilled the inclusion criteria. For data collection on risk factor profiles, addresses of spouses or close relatives of hikers and skiers who suffered sudden death were available in 314 cases. 247 questionnaires (79 %) were returned and after subsequent telephone interviews for data completion, all of them were included for analyses.

Controls: Control subjects were recruited from the population of male hikers and skiers from Austria and Germany. Within 2 consecutive summer and winter seasons, hikers and skiers were interviewed with a similar standardised questionnaire as used for cases. Inquiries were carried out on 40 frequented mountain paths and huts and in 3 Austrian ski resorts of the western part of the Austrian Alps. There, data from all male hikers and skiers over the age of 34 were recorded successively for a certain period in the morning and the afternoon. Less than 10 % refused the inquiry. Afterwards, controls were matched to the cases in terms of age, nationality, type and frequency of mountain sports activities. Three controls (n = 741) were selected for each case.

Data collection: The questionnaire employed was tested in a preceding pilot study and was revised to improve clarity and facilitate statistical analysis. This questionnaire covered demographic variables, cardiovascular risk factors, medical history, physical activity, and additionally, individual conditions at the day of death like nutrition, start of the sports activity, etc., and symptoms and circumstances of sudden death and information on resuscitation for cases. Trained interviewers were responsible for the data collections. Habitual physical activity was classified as mild to moderate and strenuous activity. Mild to moderate activity was defined as needing up to 5 metabolic equivalents (METs; 1 MET = 3.5 ml/kg/min oxygen uptake) and strenuous activities of 6 or more METs (35).

Statistics

Data are mainly presented as frequencies. Due to the study design the primary statistical approach was a case-control analysis between hikers who died suddenly during mountain hiking or downhill skiing and randomly selected controls. Differences in cardiovascular risk factors, physical activity and demographic characteristics were evaluated univariatly by Mann-Whitney, Chi-square or Fisher‘s exact tests. Logistic-regression analysis was used to estimate adjusted odds ratios and their 95% confidence intervals for cardiac death outcome. All P values were two-tailed and values below 0.05 were considered to indicate statistical significance.

RESULTS

Frequency of SCDs during a 9 year observation period

The age and gender-related numbers of SCDs during mountain hiking and downhill skiing within a 9 year period in Austria are shown in Table 1. Male hikers and skiers over the age of 40 comprise about 90 percent of all SCDs. SCDs are rare in females and young males. However, young females seem to have relatively frequent SCDs during skiing as compared with females over 40 years of age. Considering the age distribution of the male population at risk, a steep increase of the SCD risk with increasing age becomes obvious (Fig.1).

	Hikers		Skiers	
	Males	**Females**	**Males**	**Females**
Age (years)				
< 20	0	0	0	1
21-40	14	0	13	6
41-60	166	12	81	0
> 60	192	16	54	0
Total	372	28	148	7

Table 1. Age- and gender-dependent numbers of sudden cardiac deaths (SCDs) during hiking and skiing in the Austrian Alps within a 9 year observation period

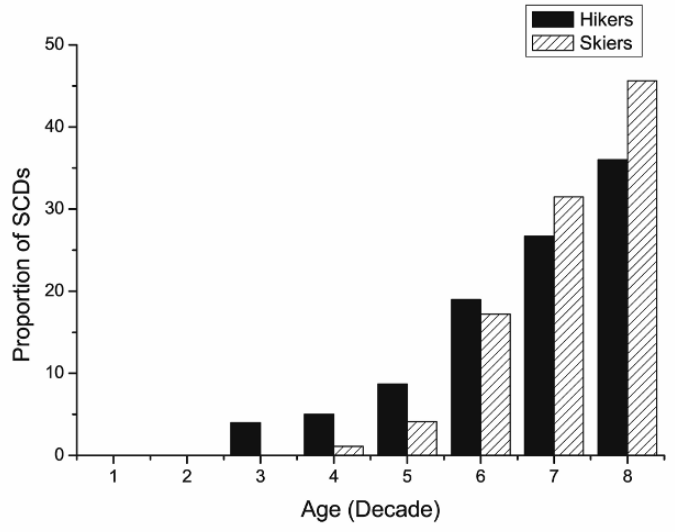

Figure 1. Age-specific proportions of SCDs in male mountain hikers and downhill skiers over the age of 34.

In comparison to the number of traumatic events, SCDs in hikers and skiers seem to be more frequent at altitudes up to 2000 m compared to those over 2000 m ($P < 0.05$), (Fig. 2, 3). The risk to suffer from SCD during hiking and skiing was greatest on the first day at altitude when 50 % of all SCDs occurred. Related to traumatic events, which are assumed to vary corresponding to the number of mountaineers, SCDs are more frequent in the late morning hours (7), (Fig. 4). The duration of activity did not influence the frequency of SCDs in hikers or skiers. However, the SCD risk clearly increased as the time from the last food and fluid intake increased. It was about 3 times higher 2 to 3 hours post intake when compared to 1 hour post intake ($P < 0.05$). Early cardio-pulmonary resuscitation was started in 33 % of skiers and in 14 % of hikers up to 5 minutes after occurrence of unconsciousness ($P < 0.05$).

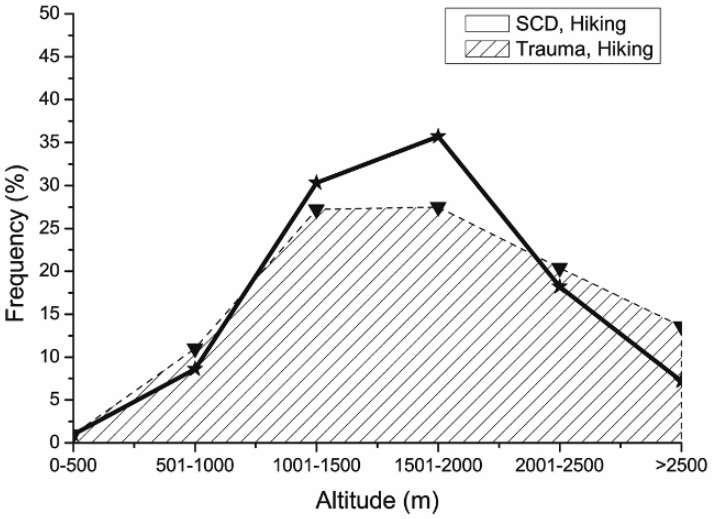

Figure 2. Altitude-dependent frequencies of SCDs and traumatic events in males > 34 years who died suddenly during mountain hiking within a 9 year period.

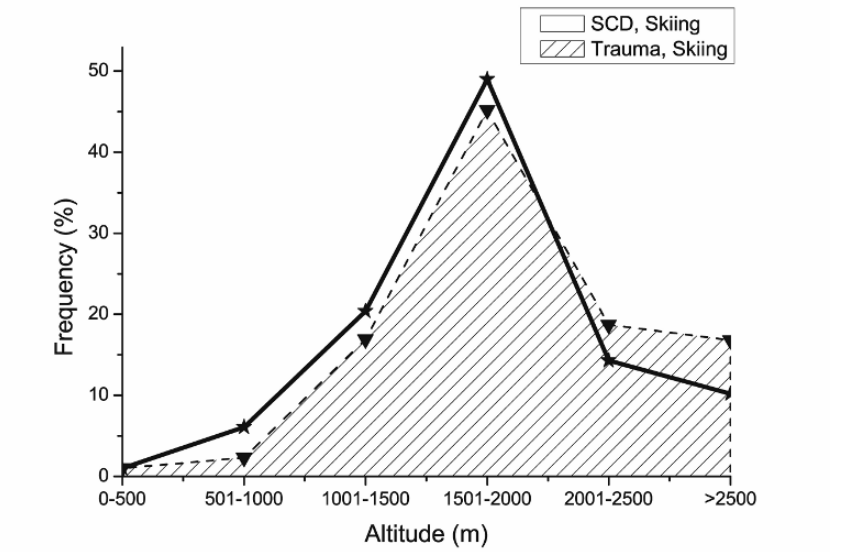

Figure 3. Altitude-dependent frequencies of SCDs and traumatic events in males > 34 years who died suddenly during downhill hiking within a 9 year period.

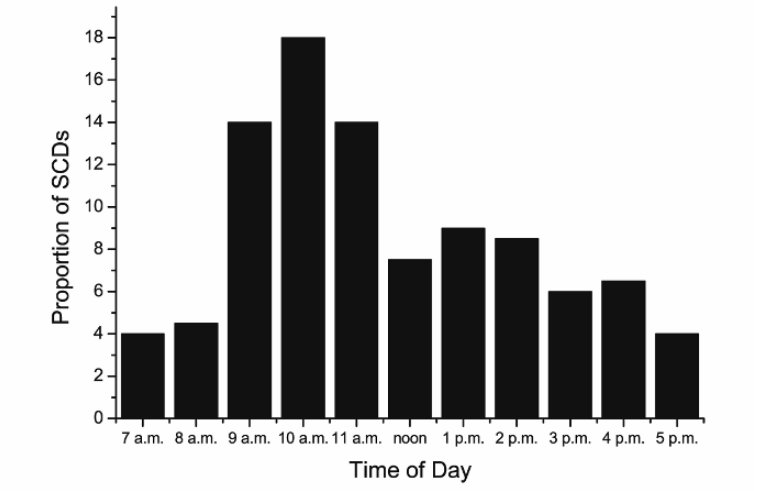

Figure 4. Day time-dependent frequencies of SCDs related to the frequencies of traumatic events in males > 34 years who died suddenly during hiking within a 9 year period.

The case-control-study

SCD risk in mountain hikers

Hikers who died suddenly during mountain hiking were much more likely to have

had a prior myocardial infarction (MI) (17 % vs. 0.9 %), known coronary artery disease (CAD) without prior MI (17 % vs. 4 %), diabetes (6 % vs. 1 %), hypercholesterolemia (54 % vs. 20 %), and were less engaged in regular mountaineering activities (31 % vs. 58 %) compared with hikers from the control group (all $P < 0.001$). Logistic regression analysis showed those 5 variables to be significantly predictive for SCD outcome during mountain hiking. Hikers with a previous MI had a 10.9 (3.8-30.9) times higher adjusted SCD risk, those with diabetes a 7.4 (1.6-34.3), those with known CAD without prior MI a 4.7 (2.4-9.2), and hikers with hypercholesterolemia a 3.4 (2.2-5.2) fold increased risk. Mountain sports activities for more than 2 weeks per year effected a marked risk reduction (0.23; 0.1-0.4), (Table 2).

Table 2. Odds ratios (95 % confidence intervals) (multivariate) regarding the prevalence of risk factors among mountain hikers and downhill skiers who suffered SCD compared to controls (hikers or skiers without SCD)

Risk factor	Hikers	Skiers
Prior MI	10.9 (3.8-30.9)	92.8 (22.8-379.1)
Known CAD	4.7 (2.4-9.2)	4.8 (1.1-21.2)
Hypertension	1.5 (0.9-2.4)	9.0 (4.0-20.6)
Hypercholesterolemia	3.4 (2.2-5.2)	0.59 (0.2-1.5)
Diabetes	7.4 (1.6-34.3)	1.1 (0.1-9.5)
High intensity exercise > 1 time per week	1.4 (0.8-2.3)	0.17 (0.04-0.7)
Mountain sports activities > 2 weeks per year	0.23 (0.1-0.4)	1.2 (0.55-2.6)

SCD risk in downhill skiers

Male skiers over the age of 34 who suffered SCD had much more frequently a prior MI (41 % vs. 1.5 %), hypertension (50 % vs. 17 %), known CAD without prior MI (9 % vs. 3 %), and were less engaged in strenuous exercise (4 % vs. 15 %) when compared to controls (all $P < 0.05$). Logistic regression analysis showed those 4 variables to be significantly predictive for SCD outcome during downhill skiing. Skiers with a previous MI had a 92.9 (22.8-379.1) times higher adjusted SCD risk, skiers with hypertension a 9.0 (4.0-20.6) and those with known CHD without prior MI a 4.8 (1.1-21.2) fold increased risk. High intensity exercise more than 1 time per week effected a marked risk reduction (0.17; 0.04-0.74), (Table 2).

DISCUSSION

There exists consensus that deaths according to the given SCD definition are mostly cardiac deaths (9, 20). Whereas the vast majority of sudden cardiac death in subjects 35 years of age and younger are known mainly to be due to structural cardiovascular disease (e.g. hypertrophic cardiomyopathy), coronary artery disease is mainly responsible for those over 35 years (12, 24). Therefore, an increasing frequency of SCDs with age is not surprising. However, data regarding the incidence of SCDs during sports activities, especially during mountain sports, are rare (4, 6, 8). The Framingham study, carried out over a 28-yr observation period, indicates an annual overall SCD-risk for persons between 35 and 70 years of age to be 2.6 per 1000 men (9). This corresponds to 1 SCD per 3,370,000 hours. In comparison, we demonstrated that the SCD-risk for men above the age of 34 when downhill skiing increases 2.2-fold (1 SCD per 1,500,000 hours) and when mountain hiking 4.2-fold (1 SCD per 802,000 hours) (4). The SCD-frequency when mountain hiking is similar when compared with cross-country skiing (1 SCD per 600,000 hours) (33) and jogging (1 SCD per 400,000 hours) (30), but markedly lower in downhill skiing. The lower SCD-risk in downhill skiing compared with mountain hiking may be caused by the different type of exertion in both sports and by the presumably different level of fitness of skiers and mountain hikers. Compared to hikers the SCD risk in male skiers increases markedly at an older age, e.g. over 60 years. This may again be related to the differences in exertion during hiking and skiing. In female skiers SCDs only occurred at an age up to 40 years, suggesting different mechanisms causing SCD at least between female hikers and skiers. The pronounced SCD risk on the first day at altitude may be attributed to the acute exposure to altitude and exercise. Our findings do not indicate that altitude itself has an important impact on SCD. Why SCDs occurred most frequent at the altitude between 1500 and 2000 m may simply be due to the greater exposure times at that altitude which is at least partly supported by the frequency distribution of traumatic events. Nevertheless, it has been shown that especially the acute exposure to moderate altitude in the elderly is associated with hypoxemia, sympathetic activation, and pulmonary hypertension which are improving during acclimatization (22). The late morning peak of SCDs in hikers, which has also been described for nonfatal myocardial infarction and transient myocardial ischemia, suggests that sudden cardiac death may be triggered by increases in adrenergic activity, systemic arterial pressure, heart rate, vascular tone, and coagulability that occur in the morning (31). Beside the small increase of the SCD risk with increasing altitude up to 2000 m in hikers and skiers a more clear risk increase exists as the time from the last food and fluid intake increases. Vigorous exertion, dehydration, depletion of carbohydrate stores, and altitude are all known to activate the sympathetic nervous system and decrease vagal activity, promoting plaque ruptures and an increase in the susceptibility to ventricular fibrillation (15, 17, 21, 22, 27, 28, 34). When SCD occurs, the rapid defibrillation and/or cardiopulmonary resuscitation (CPR) are of utmost importance but CPR has been started in 33 % of skiers and only in 14 % of hikers during the first 5 minutes after occurrence of unconsciousness and no use of electrical defibrillation has been documented. That could at least partly contribute to the lower incidence of SCDs in skiers because CPR can double or triple survival from witnessed SCD at

most intervals to defibrillation (18). Besides, the introduction of public access defibrillators in popular areas in the mountains might lead to a reduction of fatal outcome of cardiac arrest (11).

Prior MI, diabetes mellitus, known CHD without prior MI, hypercholesterolemia, and hypertension were found to be independent risk factors associated with SCD during downhill skiing and mountain hiking in males over the age of 34. Regular mountain sports activities decreased the SCD risk. The epidemiological association between these factors and the risk of all manifestations of coronary artery disease including SCD is well established. Interesting, however, is the fact that the presented risk factor profile for skiers seems to be different from that of hikers who suffered SCD (Table 2). This difference may well be related to the different types of exercise and/or environmental conditions. Whereas downhill skiing is characterized by intermittent bouts of intensive static-dynamic short term (1-3 minutes) work loads, mountain hiking means prolonged relatively uniform exertion at an intensity below the individual anaerobic threshold (5). Downhill skiing is usually performed in the winter time at low ambient temperature with relatively rapid changes in altitude and mountain hiking is performed in the summer months at comparatively warm temperature with rather slow ascents and descents. These various conditions may represent discriminative triggers for SCD. Coronary artery sclerosis is the overwhelming cause of SCD in persons > 34 years and traditional risk factors have been shown to be correlated to coronary plaque morphology. Healed MI and systemic hypertension were rather related to SCD with stable plaques and hypercholesterolemia with acute plaque rupture (32). Therefore in skiers especially non-occlusive plaques may precipitate ischemia leading to an imbalance between oxygen demand and supply and subsequent lethal arrhythmias. In contrast acute plaque rupture with thrombus formation and subsequent lethal arrhythmias may be assumed to be a more dominant mechanism precipitating SCD during hiking.

The facts that skiers took advantage of regular intense exercise and hikers from regular mountaineering underline also the importance of sport specific training for SCD prevention. Generally, habitual vigorous exertion increases basal vagal tone, resulting in increased cardiac electrical stability and in protection against ventricular fibrillation (19).

The presented findings enable identification of mountaineers at increased SCD-risk, of triggers for SCDs in the mountains, and recommendation of preventive measures, e.g. pharmacological interventions, adaptation to specific mountain activities, and adequate behavior during mountaineering. They also underline the need for a more common availability of defibrillators in the mountains and intensified cardio-pulmonary resuscitation training for all mountaineers.

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Chapter 2

BIVENTRICULAR FUNCTION AT HIGH ALTITUDE: IMPLICATIONS FOR REGULATION OF STROKE VOLUME IN CHRONIC HYPOXIA

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Abstract: The myocardium is well protected against chronic hypoxia. In chronic hypoxia stroke volume falls both at rest and on exercise. The fall in stroke volume is associated with reduction in left ventricular dimensions and filling pressure. An obvious explanation for this is the reduction in plasma volume observed at high altitude, but this does not appear to be the whole story. Neither is left ventricular systolic function abnormal even at the summit of Mount Everest. Hypoxia itself may have a direct effect on impairing myocardial relaxation. Increased pulmonary vascular resistance leads to right ventricular pressure overload. This may impair right ventricular function, and reduce stroke volume and venous return to the left atrium. Interaction between the right and left ventricles, which share a common septum and are potentially constrained in volume by the pericardium, may impair diastolic left ventricular filling as a consequence of right ventricular pressure overload, and hence reduce stroke volume. It is questionable how clinically significant is this left ventricular diastolic dysfunction. The relative importance of different mechanisms which reduce stroke volume probably depends whether hemodynamics are measured at rest or on exercise. Intervention with sildenafil to ameliorate hypoxic pulmonary vasoconstriction is associated with both an increase in exercise capacity and stroke volume in hypoxia. Whether these have a causal association remains to be demonstrated.

Key Words: pulmonary hypertension, stroke volume, right ventricle, pericardium

CARDIAC ADAPTATION TO HIGH ALTITUDE: DEFENCE AGAINST HYPOXIA

Although hypoxia is a myocardial depressant, the heart has evolved multiple defence mechanisms to protect itself from hypoxia. Data collected from high altitude residents and compared to recently acclimatized lowlanders reveals the nature of these

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protective adaptations. The adaptations include greater decreases in stroke volume during chronic as compared to recent acclimatization, which correlate with hematocrit. Chronic hypoxic exposure is associated with greater heart rate and cardiac output at maximal exercise, lower pulmonary arterial pressure (in Tibetans) (22), and greater parasympathetic activity (58). There is greater myocardial oxygen extraction manifest by an increased myocardial arteriovenous oxygen difference (PO2 of coronary sinus blood 18 Torr at 3100 m), preference of the myocardium for glucose metabolism over free fatty acids (26, 27), alterations in intracellular metabolism, and increased coronary vessel density (2) and capillary density (38), while coronary blood flow is reduced (21). There is secondary erythrocyctosis and increased tissue myoglobin as well as increased red blood cell 2,3 DPG which shifts the oxygen-hemoglobin dissociation curve to the right so facilitating release of oxygen in the tissues (35).

Adaptive changes such as the reduction in stroke volume do not return to sea level values immediately on return to sea level and in some cases may take years to normalize (49).

STROKE VOLUME AT HIGH ALTITUDE

The consequences of chronic cardiac adaptation are shown in Figure 1. In chronic hypoxia stroke volume is reduced both at rest and on exercise and this reduction is progressive with altitude (1, 32, 43). Furthermore the stroke volume is appropriate for the left ventricular filling pressure (43) and in recently acclimatized subjects is not normalized by administration of 100% oxygen. Left ventricular end-diastolic size is reduced (1, 15, 25, 50) and this is mainly determined by the reduced filling pressure, itself a consequence of reduced plasma volume associated with secondary erythrocytosis.

Reductions in heart rate and stroke volume (46) both account for the reduced cardiac output at high altitude, heart rate becoming increasingly important above 4000 m to compensate for the reduced stroke volume. The use of beta-blockers to reduce heart rate results in less reduction in stroke volume (56), as stroke volume and heart rate compensate for each other.

CARDIAC FUNCTION, PULMONARY HYPERTENSION AND VENTRICULAR INTERACTION IN CHRONIC HYPOXIA

In recently acclimatized subjects, myocardial contraction is enhanced for a given filling pressure (15). Systolic LV function is maintained in the face of chronic hypoxia to the summit of Everest (43; 50) but left ventricular relaxation is impaired and reduces early diastolic filling (9). There is also evidence for altered right ventricular diastolic function in acute studies (29).

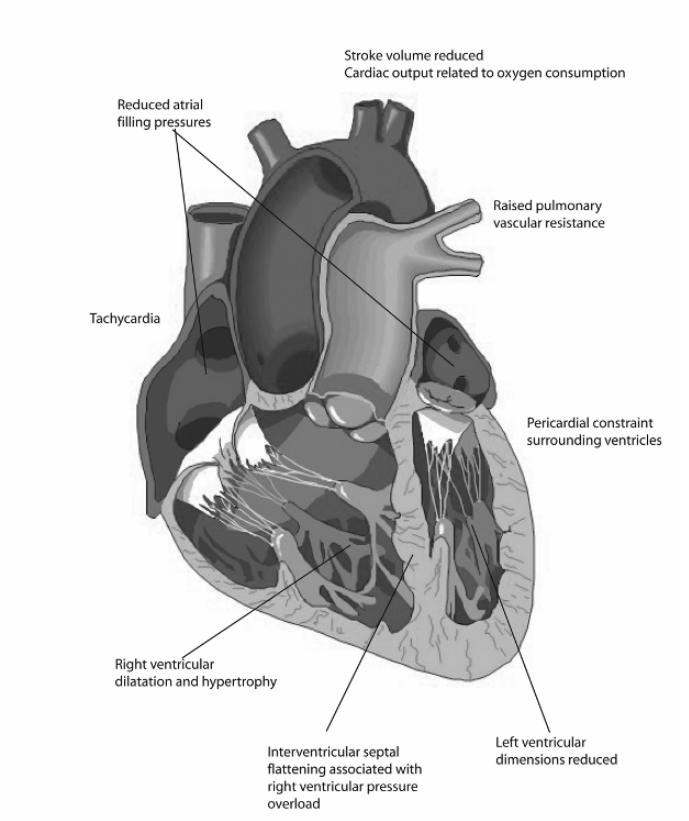

Figure 1. The effects of chronic hypoxia on the heart.

Hypoxia may directly affect left ventricular relaxation and impair early diastolic filling both chronically (19; 20) and acutely (33) as a consequence of changes in intracellular calcium handling (30). At a PO2 of 35 mm Hg myocardial stiffness is increased (19). Raised levels of catecholamines potentiate hypoxia-induced impairment of ventricular relaxation because of increased myocardial energy requirements (6). Such a direct effect of hypoxia has recently been confirmed in mice to be caused by reduced phospholamban phosphorylation (34).

In patients with COPD, another model of chronic hypoxia, abnormalities of left ventricular diastolic function are common (90%) (10; 48) although systolic dysfunction is uncommon (5%) (53). The effect of hypoxia on left ventricular relaxation has been shown to be blocked by respiratory acidosis (7; 20) which improves relaxation.

In some subjects at high altitude the effects of brisk acute pulmonary vasoconstriction, especially during exercise, may exert a major influence on cardiac function (Figure 1). This results in an increased pulmonary vascular resistance which typically increases by about 50% when PaO2 falls to 50 Torr (23).

In response to raised afterload the right ventricle must develop higher pressures than normal. It becomes dilated with increased end-diastolic volume and develops concentric hypertrophy (42). The consequent pulmonary hypertension acutely increases right ventricular diastolic pressure, induces tricuspid regurgitation, increases right ventricular isovolumic relaxation, and decreases right ventricular filling time (Figure 2) which reduces right ventricular stroke volume and venous return to the left ventricle.

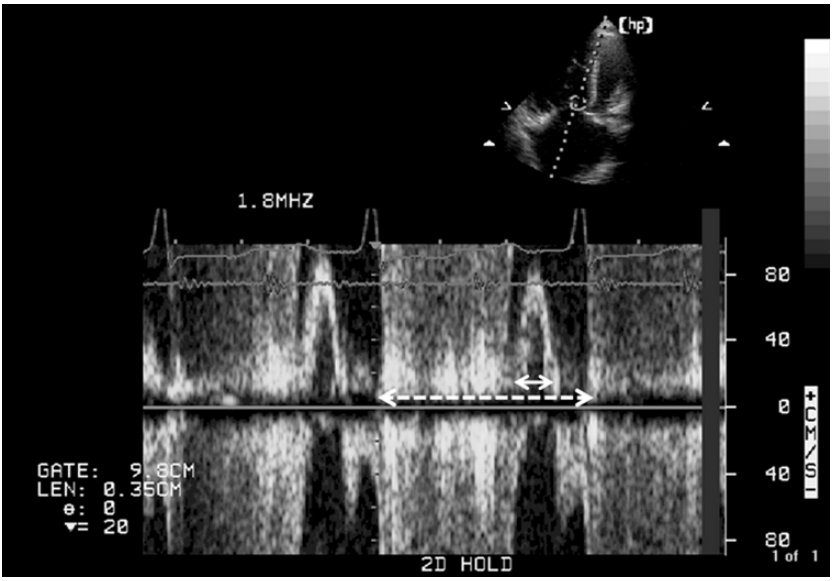

Figure 2. Pulsed Doppler echocardiogram showing limitation of right ventricular filling time caused by tricuspid regurgitation in pulmonary hypertension. ECG and phonocardiogram are shown above the Doppler recording. The solid arrow indicates the brief period of right ventricular filling compared to the length of the cardiac cycle (dotted arrow).

Because of the anatomical and mechanical association between the ventricles, the volume of one ventricle can directly affect the volume and pressure within the other ventricle. This relationship was originally described by Henderson and Prince in 1914 (24) who showed that the left ventricular pressure-volume relationship was altered by right ventricular filling, the septum behaving as a passive compliant membrane between the two chambers. This ventricular interaction has been shown to play a role in left ventricular diastolic dysfunction in hypoxic patients with COPD (10).

In the face of pulmonary hypertension the geometry of the right ventricle is altered as it assumes a more spherical shape and interventricular septal flattening occurs (Figure 3). This is associated with a decrease in left ventricular volume and a leftward shift of the septum (3; 17; 31; 41). The end-diastolic septal position is determined by transseptal pressure difference (31). Normally this is about 3 mm Hg at end-diastole with the septum convex to the right ventricle. Right ventricular pressure overload reduces or reverses the septal pressure difference (14; 51).

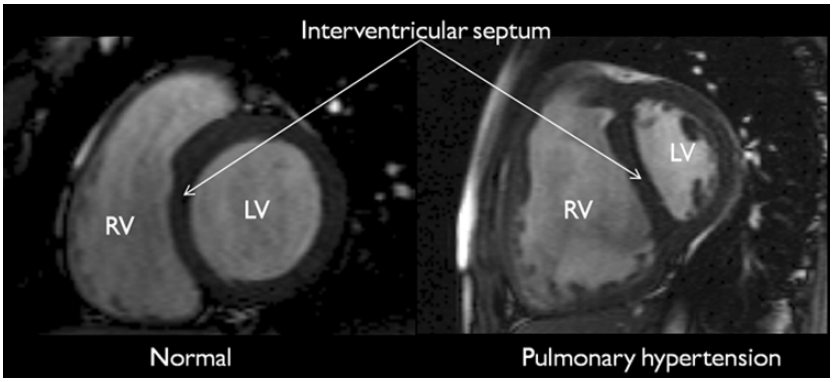

Figure 3. Interventricular septal shift in a normal subject and a patient with pulmonary hypertension. Note the enlarged right ventricle which is a consequence of pulmonary hypertension.

Figure 4 demonstrates the right and left ventricular interaction in pulmonary hypertension. Right ventricular systole becomes prolonged as its ejection time increases because of increased afterload. Early diastole in the left ventricle now coincides with late right ventricular systole, a time when the interventricular septum is deviated to the left. This impairs early left ventricular filling and results in reduced left ventricular volumes which themselves reduce stroke volume (36). The net effect of raised pulmonary arterial pressure on left ventricular diastolic function is shown in Figure 5 where the transmitral E/A ratio was used as a measure of diastolic dysfunction.

These studies show that the septum flattens only when trans-septal pressure (left ventricular pressure - right ventricular pressure) is about -5 mm Hg (13). The quantitative relationship between right ventricular pressure overload and left ventricular diastolic function has been assessed in chronic pulmonary hypertension. Only patients with a systolic pulmonary artery pressure $\geq$ 60 mm Hg exhibit altered left ventricular filling pattern and this is caused by impaired relaxation (39). Seventy percent of patients with systolic pulmonary arterial pressure $\geq$ 60 mm Hg had interventricular septal flattening in early diastole. This means that clinically important ventricular interaction does not apply to most people exposed to chronic hypoxia at rest but may become important on exercise in some (4).

Pericardial constraint also mediates shifts in the left ventricular diastolic pressure volume relationship when the pericardium is dilated beyond its unstressed volume to a point when any change in volume causes a change in pericardial pressure, such as increases in right ventricular or atrial volume. The reduced left ventricular volume will tend to offset this. Pericardial constraint shifts the left ventricular pressure volume relationship upwards (44). In this sense pericardium has similar properties to a paper bag which is being inflated: a very flat compliance curve up to an inflection point after which it is very steep. Pericardium constraint is not important at rest in the chronically right ventricular pressure overloaded heart since the pericardium adapts to cardiac enlargement (8). The pericardium is only likely to be important on exercise if right ventricular dilatation is large (37). Pulmonary constraint may also compress the heart because of increased lung volumes seen at altitude although this remains to be shown.

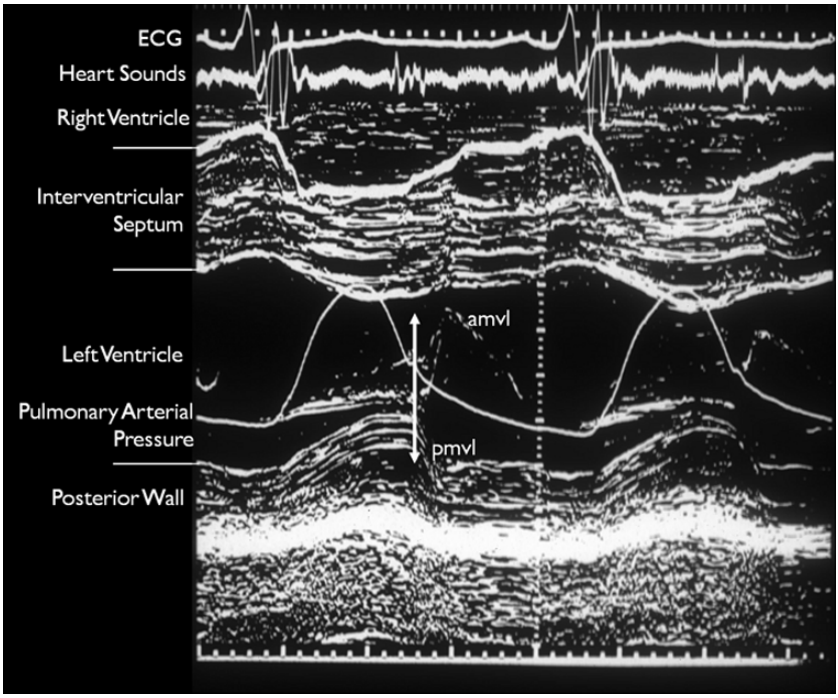

Figure 4. M-mode echocardiogram showing evidence for raised right ventricular pressure at the time of mitral valve opening. A simultaneous micromanometer-tipped catheter pressure tracing from the pulmonary artery has been superimposed on the recording. This closely reflects right ventricular pressure during systole. The vertical arrow shows the point of mitral valve opening (onset of left ventricular filling) which occurs when pulmonary arterial (and right ventricular) pressure are falling but still significantly elevated. The consequence is impairment of early diastolic filling of the left ventricle. Amvl, anterior mitral valve leaflet; pmvl, posterior mitral valve leaflet.

The effect of significantly increased right ventricle afterload is to increase myocardial oxygen demand and wall stress which impairs myocardial perfusion and may even cause myocardial ischemia (11). Note that wall stress is reduced by the compensatory right ventricular hypertrophy. Nelson et al have demonstrated substantial circumferential compressive stress (- 20 mm Hg) in the septum during flattening and inversion (40). They speculate that this is sufficient to cause myocardial ischemia despite normal coronary arteries. Left bundle branch block which alters septal contraction pattern has been shown to cause septal hypoperfusion on thallium scanning possibly by a similar mechanism, although this has been challenged since there is an associated reduction in myocardial work in left bundle branch block (52).

Where raised coronary venous pressure occurs as a result of increasing right atrial and right ventricular pressure, left ventricular end-diastolic pressure is increased despite no change in left ventricular volume (55). This is possibly related to increased intramyocardial blood volume and myocardial edema (12) which increase myocardial wall stiffness. Another potential mechanism of left ventricular diastolic dysfunction in

this setting is left ventricular asynchrony (5).

It has previously been suggested that the normal pulmonary capillary wedge pressure supports the absence of diastolic left ventricular dysfunction. This cannot be substantiated since in severe idiopathic pulmonary arterial hypertension when LV function is significantly impaired pulmonary capillary wedge is usually normal. The effect of impaired diastolic filling on the pulmonary capillary wedge pressure may be masked by the reduced left ventricular volume.

The significance of diastolic impairment of left ventricular function will only be effectively assessed by recording left ventricular pressure-volume loops in chronic hypoxia.

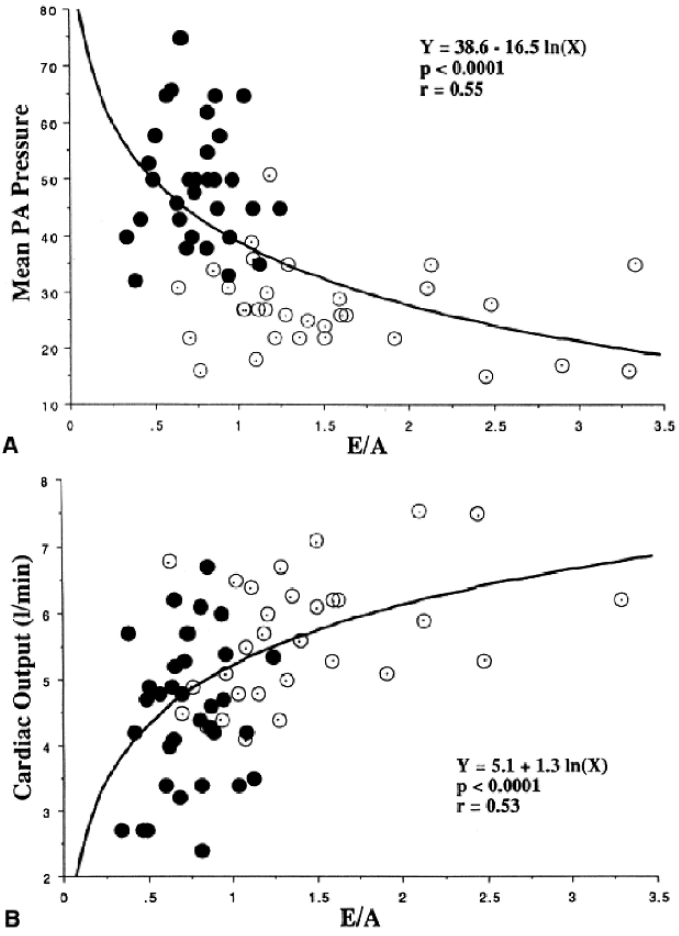

Figure 5. Effect of pulmonary hypertension on left ventricular diastolic function and cardiac output according to Mahmud 2002, and collected in patients before and after pulmonary endarterectomy demonstrating a wide spectrum of pulmonary hypertension. (A) Inverse correlation between transmitral E/A ratio (E/A) and mean pulmonary artery (PA) pressure. (B) Direct correlation between transmitral E/A ratio (E/A) and cardiac output (CO). Filled circle: pre-pulmonary thromboendarterectomy; open circle: post-pulmonary thromboendarterectomy.

CAN PREVENTION OF HYPOXIC PULMONARY VASOCONSTRICTION ALTER STROKE VOLUME IN CHRONIC HYPOXIA?

Sildenafil inhibits hypoxic pulmonary vasoconstriction by inhibiting phosphodiesterase 5, an enzyme present in high concentrations in the pulmonary circulation and responsible for c'GMP hydrolysis in the lung (57). This prolongs the vascular relaxant and antiproliferative properties of nitric oxide. Sildenafil is used for this purpose as a treatment for idiopathic pulmonary arterial hypertension (16).

The administration of sildenafil in lowlanders who ascended to Everest base camp at 5245 m has been investigated in a double-blind placebo-controlled crossover study in 14 recently acclimatized healthy subjects (18). This study showed increased maximum exercise workload and maximum cardiac output at 5245 m. At peak exercise at low altitude sildenafil increased median stroke volume from 71 to 82 ml and at 5245 m from 91 to 102 ml. The estimated systolic pulmonary arterial pressure at peak exercise at 5245 m was 34 mm Hg on placebo and 27.5 mm Hg on sildenafil. The study could not demonstrate a causal relationship between stroke volume or cardiac output, improvement in hemodynamics and exercise capacity (47). Sildenafil limits the hypoxia of high altitude and does not interfere with acclimatization (45).

Similar improvements in cardiovascular function were obtained in acute hypoxia at 3874 m during submaximal exercise and showed a robust association between the fall in exercise stroke volume from sea level to high altitude and improvement in exercise performance (28).

CONCLUSIONS

A single cause for the reduced stroke volume in chronic hypoxia has never been satisfactorily demonstrated. In 2000 Wagner reviewed potential cardiac and non-cardiac causes and favoured the explanation that the reduced stroke volume was a passive consequence of reduced demand from the tissues (54).

The heart is well protected against chronic hypoxia but not against the consequences of brisk pulmonary vasoconstriction. The clinical significance of ventricular interaction and consequent diastolic left ventricular dysfunction in reducing stroke volume in chronic hypoxia is not clear since of the many hemodynamic measurements made at high altitude a minority fall into a range which would be likely to have a clinically significant effect. The most likely time when such interactions would occur is during exercise or HAPE when the pulmonary circulation is maximally stressed. Studies to elucidate diastolic dysfunction of the left ventricle have not been conducted in long-term chronic hypoxia. The ideal studies which need to be performed to measure pressure volume relationships are invasive and difficult to undertake. Nevertheless the recent studies using sildenafil provide tantalizing new evidence that perhaps relief of hypoxia-induced pulmonary hypertension may lead to a beneficial improvement in stroke volume.

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Chapter 3

CONTROL OF CORONARY BLOOD FLOW DURING HYPOXEMIA

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Abstract: Coronary vascular resistance is regulated by a variety of factors including arterial pressure, myocardial metabolism, autonomic nervous system as well as arterial O_2 tension (hypoxia). Progressive hypoxemia results in graded coronary vasodilation that is significantly more pronounced when arterial O_2 tension falls below 40 mmHg. Microvascular studies have demonstrated that O_2 has direct effects on vascular smooth muscle likely mediated by O_2 sensors located in vessels < 15 μm diameter. Recent data indicates that hypoxia-induced inhibition of the pentose phosphate pathway and the subsequent decreases in NADPH and intracellular Ca^{2+} represent an important O_2 sensing mechanism in vascular smooth muscle. However, *in vivo* experiments suggest direct microvascular effects of O_2 contribute little to hypoxic coronary vasodilation. The vasodilation is mediated, in part, by local vasoactive metabolites produced in proportion to the degree of hypoxemia, reflex-mediated increases in myocardial metabolism and diminished myocardial tissue oxygenation. In particular, production of adenosine has been shown to increase exponentially with the degree of hypoxia and blockade or degradation of adenosine markedly impairs hypoxia-induced coronary vasodilation. Other investigations support the role of endothelial derived relaxing factors (nitric oxide, prostacyclin) in control of coronary blood flow during hypoxia. Additionally, reductions in PO_2 hyperpolarize coronary vascular smooth muscle via K^+_{ATP} channels which represent important "end effectors" that significantly contribute to hypoxic coronary vasodilation. Taken together, these data indicate that the coronary vascular response to hypoxia depends on metabolic and endothelial vasodilatory factors that are produced in proportion to the degree of hypoxemia and that function through mechanisms depending on K^+_{ATP} channels.

Key Words: coronary circulation, arterial oxygen tension, adenosine, nitric oxide, K^+_{ATP} channels

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The coronary circulation has a remarkable ability to balance coronary blood flow with myocardial metabolism under a variety of physiologic/pathophysiologic conditions, including reductions in arterial PO_2, i.e. hypoxemia (28; 87; 88). This balance between O_2 delivery and metabolism is critical since the myocardium has a very limited anaerobic capacity, thus the heart is highly dependent on a continuous supply of O_2 from the coronary circulation to meet its metabolic requirements (21; 87; 88). If this need for O_2 is not met, the resulting underperfusion substantially diminishes cardiac function within seconds (7; 41; 42; 81). Thus, tight control of coronary blood flow is essential to maintain myocardial performance.

Hypoxemia was first shown to induce dramatic increases in coronary blood flow in the 1920s by Hilton and Eichholtz (44) and by Gremels and Starling (37). This observation has since been confirmed by numerous other laboratories. These early studies suggested that the decrease in coronary vascular resistance to hypoxemia was mediated by direct action of low arterial PO_2 on vascular smooth muscle. However, additional studies over the years have shown that control of coronary blood flow during hypoxemia is much more complex and depends on production of metabolic and endothelial factors that reduce coronary vasomotor tone in proportion to the degree of hypoxia. The purpose of this review is to outline the mechanisms by which hypoxemia regulates coronary vascular resistance and the balance between myocardial O_2 delivery and metabolism.

CORONARY, AUTONOMIC AND CARDIOVASCULAR RESPONSE TO HYPOXEMIA

Progressive decreases in arterial PO_2 (hypoxemia) result in graded coronary vasodilation that is significantly more pronounced when arterial PO_2 falls below ~ 40 mmHg (Figure 1) (5; 6; 18; 28; 72; 91). This phenomenon is clearly evident even from the early the data of Gremels and Starling (37). Similarly, coronary venous PO_2, an index of myocardial tissue PO_2, is maintained constant at normal levels (~ 20 mmHg) until this critical arterial PO_2 is reached (Figure 1) (72). These findings indicate that the increases in coronary blood flow with more moderate levels of hypoxia are adequate to meet the myocardial requirements for O_2 thus maintaining the balance between myocardial O_2 delivery and metabolism. However, as arterial PO_2 is reduced below 40 mmHg, reflex activation of the sympathetic nervous system increases in aortic pressure, heart rate (Figure 2) and myocardial O_2 consumption (MVO_2; Figure 1) (30; 78); which reduces myocardial tissue PO_2 and activates powerful local metabolic vasodilator mechanisms (87). Importantly, this vasodilation alone is not sufficient to balance O_2 delivery with MVO_2 as evidenced by the increase in myocardial O_2 extraction (decrease in coronary venous PO_2, Figure 1) with arterial $PO_2 < 40$ mmHg. Evidence of cardiac underperfusion/ischemia is evident as arterial PO_2 is reduced below 30 – 35 mmHg with the onset of net lactate production, ST segment changes, diminished cardiac contractile function as well as limitation of MVO_2 (10; 78; 95).

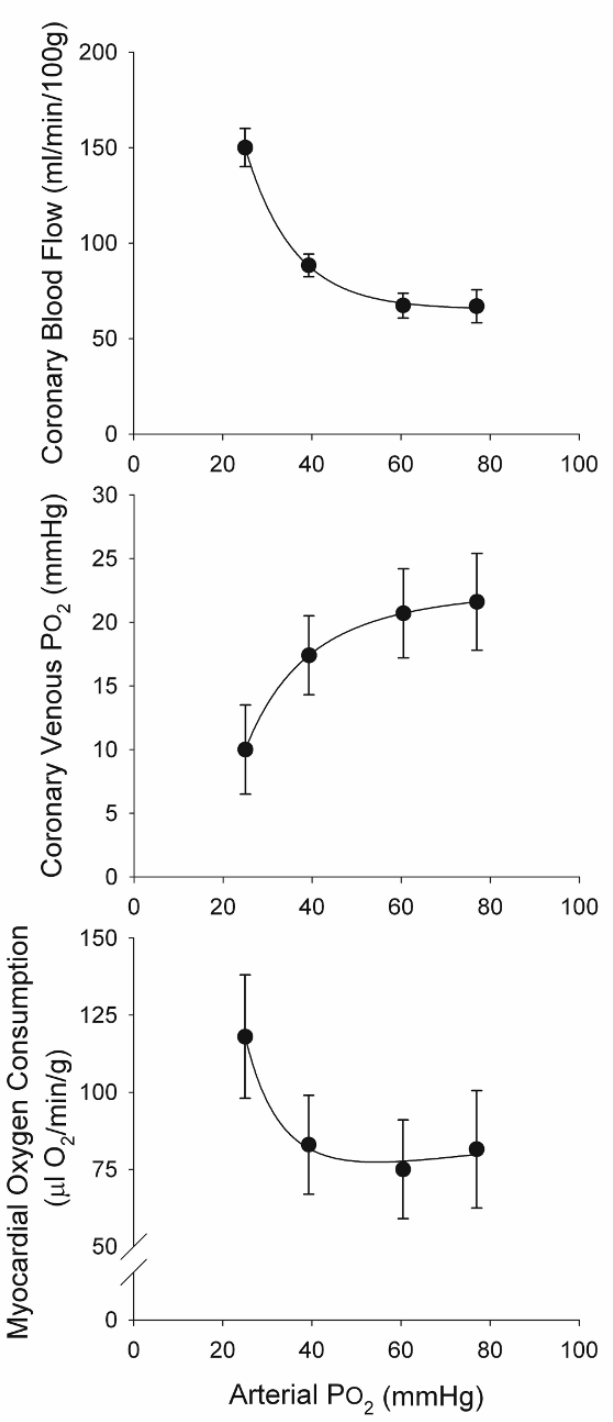

Figure 1: Effect of arterial hypoxemia on left circumflex coronary blood flow, coronary venous (sinus) PO_2 and myocardial O_2 consumption. Experiments were conducted in conscious, chronically instrumented dogs placed in a plexigas chamber in which N_2 was introduced to progressively reduce chamber O_2. Progressive hypoxemia results in graded coronary vasodilation that is significantly more pronounced when arterial PO_2 falls below ~ 40 mmHg. This vasodilation alone is not sufficient to balance O_2 delivery with increases in myocardial O_2 consumption as evidenced by the decrease in coronary venous PO_2 (increase in myocardial O_2 extraction) with arterial PO_2 < 40 mmHg.

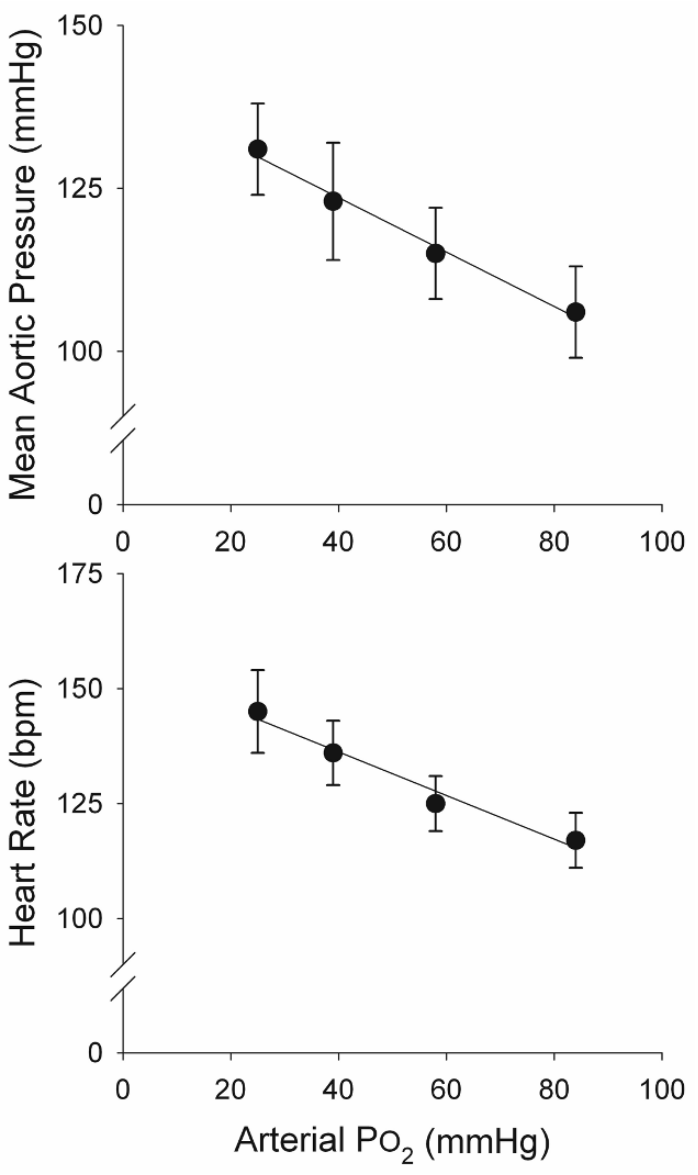

Figure 2: Effect of arterial hypoxemia on mean aortic pressure and heart rate. Experiments were conducted in conscious, chronically instrumented dogs placed in a plexigas chamber in which N_2 was introduced to progressively reduce chamber O_2. Hypoxemia results in a reflex activation of the sympathetic nervous system that progressively increases aortic pressure and heart rate as arterial PO_2 is diminished.

Sympathetic activation with hypoxemia could also contribute to control of coronary blood flow as both α- and β-adrenoceptors both directly influence vasomotor tone (36; 70; 87). Herrmann and Feigl nicely demonstrated that much of coronary vasodilation to hypoxemia is dependent on adrenergic activation as coronary blood flow increased from 0.68 ± 0.003 ml/min/g (normoxic) to 2.4 ± 0.43 ml/min/g (hypoxic: F_IO_2 = 7-10%) in adrenergically intact animals and from 0.56 ± 0.01 ml/min/g (normoxic) to

0.91 ± 0.07 ml/min/g (hypoxic: F_IO_2 = 7-10%) in adrenergically (α + β adrenoceptors) blocked animals (43). Another investigation by Folle and Aviado found that hypoxic coronary vasodilation was reduced by β-adrenoceptor blockade (32), although this was not observed in other investigations (26; 60). The decrease in hypoxic coronary vasodilation with adrenergic blockade is likely mediated by the significant attenuation of increases in MVO_2, i.e. the loss of the stimulus for local metabolic coronary vasodilation (17; 43). Data from Doherty and Liang support this hypothesis in that reductions in coronary blood flow in adrenergically blocked awake dogs correlated nicely with decreases in MVO_2 (17). Alternatively, reflex sympathetic activation has also been shown to activate α_1-adrenoceptor mediated vasoconstriction that diminishes coronary responses to systemic hypoxemia (19). This paradoxical constriction could help maintain blood flow to the vulnerable subendocardium during severe hypoxemia (45). The postulated mechanism is that release of norepinephrine during increases in sympathetic activity, i.e. hypoxemia, stimulates α-adrenoceptor vasoconstriction of medium sized intramyocardial vessels (diameter > 100 μm), which decreases intramyocardial vascular capacitance and wasteful antigrade-retrograde flow oscillations during the cardiac cycle (87,88). Whether this hypothesis is true under conditions of hypoxemia has not been investigated. Additional studies are needed to more directly assess the role of the autonomic nervous system in control of coronary blood flow during hypoxia.

O_2 AND CONTROL OF CORONARY BLOOD FLOW

In 1925, Hilton and Eichholtz proposed that reductions in coronary vascular resistance produced by hypoxemia are mediated by the direct effects of low arterial PO_2 on the microvasculature (44). This hypothesis is substantiated by studies in isolated vessel preparations which demonstrate robust vascular responses in small arteries and arterioles to changes in PO_2 (9; 33; 46; 48; 50; 69). In addition, lowering perfusate PO_2 increases coronary blood flow in isolated heart preparations (12; 74; 94). These data indicate that there are local O_2 sensing pathways that regulate coronary vasomotor tone in response to hypoxia. The work of Jackson and Duling suggest that the O_2 sensors are located in terminal arterioles, capillaries, venules (vessels < 15 μm diameter) and/or in the parenchyma; and that these sensors detect changes in PO_2 and initiate a conducted vasodilatory response from the sensor to distant arterioles (resistance vessels) (46; 48). It is postulated that this microvascular vasodilatory response involves K^+_{ATP} channels as reductions in PO_2 have been shown to increase glibenclamide sensitive K^+ current in coronary smooth muscle cells (11; 12; 33) (see Role of K^+ channels in hypoxic coronary vasodilation below). Although these data nicely demonstrate the direct effects of PO_2 on the coronary microcirculation, the exact contribution (i.e. in vivo relevance) of PO_2 to hypoxic coronary vasodilation remains unclear.

Data from Downey and colleagues suggest that regulation of coronary blood flow is closely associated with myocardial tissue PO_2 (coronary venous PO_2) irrespective of the stimulus that alters tissue oxygenation. This effect was evidenced by the fact that similar values of coronary vascular resistance were obtained whether coronary venous

PO_2 was altered by changes in coronary pressure or arterial PO_2 (20). This hypothesis is also supported by data which demonstrate that hypoxic coronary vasodilation is markedly more pronounced at low arterial PO_2 (< 40 mmHg) (5; 6; 18; 28; 72; 91).

It should be pointed out that alternative vascular O_2 sensing mechanisms that could contribute to hypoxic coronary vasodilation have recently been identified. Studies have demonstrated that red blood cells progressively release ATP with decreases in PO_2, independent of changes in pH or PCO_2 (3; 16; 24; 25). Once released, this ATP (or metabolites) acts on endothelial purinergic ($P2Y_1$) receptors to initiate a conducted vasodilatory response (23; 27; 35). Although a consistent and intriguing hypothesis, the involvement of this pathway in hypoxic coronary vasodilation has yet to be directly examined. Additionally, recent data from the Wolin laboratory reveal a novel mechanism of hypoxic coronary vasodilation in which lowered PO_2 causes a metabolic stress on the pentose phosphate pathway that results in diminished cytosolic NADPH and glutathione concentrations, decreased Ca^{2+} influx and accelerated activity of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) pump (39; 40). Thus, inhibition of the pentose phosphate pathway, which is mediated by hypoxic-induced depletion of glucose-6-phosphate (substrate for rate-limiting enzyme of the pathway), could be an important O_2 sensing mechanism in vascular smooth muscle that could contribute to control of coronary vasomotor tone during hypoxia (58).

ADENOSINE AND LOCAL METABOLIC CONTROL OF CORONARY BLOOD FLOW DURING HYPOXEMIA

Berne argued as early as 1957 that the lack of significant coronary vasodilation to hypoxemia above a coronary venous O_2 content of 5.5 ml/100 ml (PO_2 of ~ 20 mmHg) supports the role for a myocardial metabolic mechanism rather than the direct vascular effects of PO_2 as the critical variable for control of coronary blood flow (6). This observation later lead to Berne's seminal paper on the role of cardiac nucleotides in hypoxia (4). In this study, Berne found that cardiac hypoxia resulted in a decrease in coronary vascular resistance that was closely correlated with the release of significant amounts of inosine and hypoxanthine (metabolites of the potent vasodilator adenosine) from isolated cat hearts and from intact hearts of open-chest dogs. Based on these data Berne proposed the adenosine hypothesis which hypothesized that reduction of arterial PO_2 and/or increases in MVO_2 decrease myocardial tissue PO_2 thereby stimulating the breakdown of intracellular adenine nucleotides and the release of adenosine from cardiomyocytes. The resulting increase in cardiac interstitial adenosine concentration relaxes coronary arteriolar vascular smooth muscle thereby increasing coronary flow and O_2 delivery which would act to restore myocardial tissue PO_2 toward a normal level in a classic negative feedback manner.

The adenosine hypothesis has been examined by countless studies over the past 40 years (29; 87). Although data have demonstrated that adenosine is not required for local metabolic control of coronary blood flow under normal physiological increases in myocardial metabolism (2; 15; 87; 89); adenosine does contribute to control of coronary

blood flow when the myocardium becomes underperfused/ischemic, i.e. O_2 delivery is not sufficient to meet myocardial requirements for O_2 (59; 87). Thus, the imbalance between myocardial O_2 supply and demand which develops with more severe degrees of hypoxemia supports the role of adenosine as a mechanism of hypoxic coronary vasodilation. Several investigations have established that myocardial adenosine release increases exponentially with the severity of hypoxia (13-15; 43; 86; 90). The concentrations of adenosine released during hypoxia were shown to be vasoactive in bioassay experiments in isolated guinea pig hearts (83; 85). Furthermore, administration of adenosine receptor blocking drugs or enzymatic degradation of adenosine significantly reduces hypoxic coronary vasodilation in isolated hearts (55; 74; 96) and in anesthetized animals (34; 61; 67; 68). The decrease in coronary vasodilation with adenosine inhibition was consistently between 20 – 30% and determined under conditions of severe hypoxia (arterial PO_2 values between 20 - 35 mmHg). Taken together, these studies indicate that adenosine contributes to, but is not entirely responsible for, the marked hypoxic coronary vasodilation observed when arterial PO_2 falls below 40 mmHg.

ENDOTHELIAL CONTROL OF CORONARY BLOOD FLOW DURING HYPOXEMIA

The endothelium is a single layer of cells lining the luminal surface of blood vessels that provides the critical interface between blood and tissues (92). Endothelial cells produce and secrete numerous compounds that regulate a variety of (patho) physiological processes including coagulation, inflammation, permeability, cell adhesion, and vasomotor tone (31; 93). Numerous studies have established that release endothelial-relaxing and constricting factors are critical to the regulation of coronary vascular resistance (87; 88). Therefore, based on the numerous vasoactive agents released by the endothelium as well as the myocardium, it is evident that control of coronary blood flow involves a highly integrated process that does not simply rely on a single mediator (56; 66; 87).

Hypoxia has been shown to increase the release of the endothelial derived relaxing factor nitric oxide (NO) via activation of constitutive nitric oxide synthase in cultured porcine coronary endothelial cells (97). The increase in NO production with hypoxia was greater in nonresistance, epicardial coronary arteries (relative to resistance arterioles) (51); which is consistent with *in vivo* data showing a greater role for NO-mediated vasodilation in large coronary arteries (87). The role of NO in control of coronary blood flow during hypoxia is evidenced by studies which found that denudation or inhibition of nitric oxide synthase significantly attenuated hypoxic vasodilation in isolated coronary arteries (49; 64), isolated guinea pig hearts (8; 77) as well as in conscious animals (1; 65). In conscious, chronically instrumented dogs, our laboratory documented that NO synthesis blockade significantly decreased right ventricular O_2 delivery during graded systemic hypoxia (65). The reduction of coronary blood flow and O_2 delivery was similar at any given arterial PO_2 or MVO_2 indicating that the contribution of NO is not dependent on the degree of hypoxia, i.e. tonic regulation of coronary vascular tone.

In addition, NO was not essential for myocardial O_2 supply/demand balance as there was no evidence of underperfusion/ischemia in that lactate uptake was unaffected by hypoxia.

Similarly, hypoxia has also been shown to increase the release of endothelial prostaglandins, in particular prostacyclin (PGI_2), from isolated, buffer-perfused hearts (9; 75; 76). Inhibition of prostaglandin production by blockade of cyclooxygenase also diminishes hypoxic induced coronary vasodilation (9; 52; 53; 71; 74; 76; 79); however this effect is not a consistent finding (62; 63). Importantly, the effects of cyclooxygenase blockade on hypoxic coronary vasodilation *in vivo* have not been examined. The discrepancy between these studies is likely related to differences in species and experimental preparations studied (38), however it could also represent the contribution of endothelial derived hyperpolarizing factor(s) in hypoxic-induced coronary vasodilation (62; 63). Taken together, these studies strongly support the role of endothelial derived vasodilatory compounds in control of coronary vascular tone in systemic hypoxia.

ROLE OF K^+ CHANNELS IN HYPOXIC CORONARY VASODILATION

When K^+ channels open, K^+ moves down its electrochemical gradient, E_m hyperpolarizes, and smooth muscle relaxes to produce vasodilation. Conversely, when K^+ channels are inhibited by vasoconstrictors or pharmacological agents, K^+ efflux is attenuated, E_m depolarizes, L-type Ca^{2+} channel activity increases, and the intracellular Ca^{2+} concentration climbs (47). Four major classes of K^+ channels are known to be expressed in coronary vascular smooth muscle including: 1) voltage-dependent (K_V); 2) Ca^{2+}-activated (K_{Ca}) 3) ATP-sensitive (K_{ATP}); and 4) inward rectifier (K_{IR}) K^+ channels. Although the contribution of each of these channels to the regulation of coronary vasomotor tone has not been clearly defined, K^+ channels are extremely important "end-effectors" in control of coronary blood flow.

The role of K^+ channels, in particular K_{ATP} channels, in hypoxic coronary vasodilation has been extensively studied. Reductions in PO_2 hyperpolarize endothelium-intact as well as denuded coronary arteries/arterioles that is directly related to an increase in glibenclamide sensitive K^+ current (11; 12; 33; 69). Importantly, several studies have documented that blockade of K_{ATP} channels significantly impairs coronary vasodilation to hypoxia in isolated coronary arterioles, arteries and in isolated heart preparations (54; 55; 62; 63; 69; 73; 74), indicating K_{ATP} channels directly contribute to hypoxic vasodilation. However, this effect also reflects the involvement of K_{ATP} channels in adenosine-mediated vasodilation (22; 57; 84). Although the role of K_{ATP} channels in hypoxic coronary vasodilation has not been directly examined *in vivo*; these studies indicate that K_{ATP} channels are important mediators of the coronary vascular response to hypoxemia.

Alternatively, the contribution of other K^+ channels to hypoxic coronary vasodilation has not been systemically investigated. One study by Lee et al. found that blockade of K_{Ca} channels with apamin or iberiotoxin had no effect on hypoxic dilation of isolated

rabbit left circumflex coronary arteries (62). However, it should be pointed out that activation of voltage-dependent K_v channels could be important mechanism during hypoxia as these channels were recently found to significantly contribute to the balance between coronary blood flow and myocardial metabolism during increases in MVO_2 (80; 82). Additional studies are needed to more completely characterize the contribution of K^+ channels to control of coronary blood flow during hypoxia.

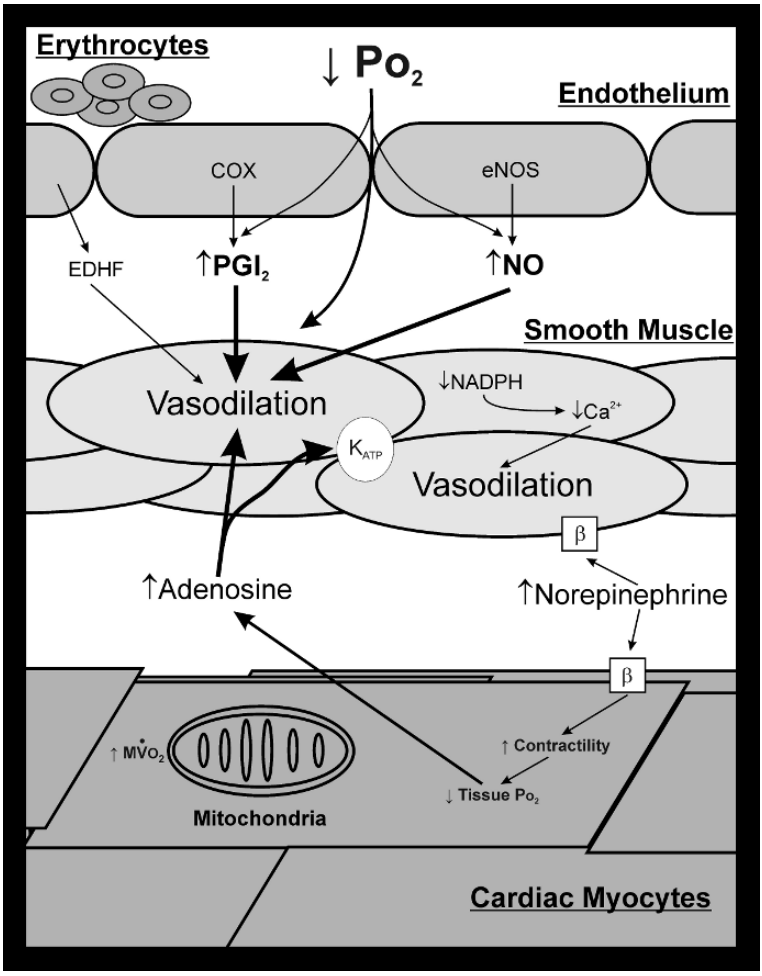

Figure 3: Schematic diagram outlining proposed mechanisms of hypoxic coronary vasodilation. The coronary vascular smooth muscle response to hypoxia depends largely on metabolic and endothelial vasodilatory factors that are produced in proportion to the degree of hypoxemia that function, at least in part, through mechanisms dependent on activation of K^+_{ATP} channels.

In summary, hypoxemia results in marked coronary vasodilation that is significantly more pronounced when arterial O_2 tension falls below 40 mmHg. The mechanisms re-

sponsible for hypoxia-induced increases in coronary blood flow have been the subject of investigations for many years and likely involve multiple mediators from various sources. A schematic diagram of proposed mechanisms regulating coronary vasomotor tone during hypoxia is shown in Figure 3. Microvascular studies have demonstrated that O_2 has direct effects on vascular smooth muscle; likely mediated by O_2 sensors located in vessels < 15 μm diameter. Recent data indicates that hypoxia-induced inhibition of the pentose phosphate pathway and the subsequent decreases in NADPH and intracellular Ca^{2+} represent an important O_2 sensing mechanism in vascular smooth muscle. However, *in vivo* experiments suggest direct microvascular effects of O_2 contribute little to hypoxic coronary vasodilation. The vasodilation is mediated, in part, by local vasoactive metabolites produced in proportion to the degree of hypoxemia, reflex-mediated increases in myocardial metabolism and diminished myocardial tissue oxygenation. In particular, production of adenosine has been shown to increase exponentially with the degree of hypoxia and blockade or degradation of adenosine markedly impairs hypoxia-induced coronary vasodilation. Other investigations support the role of endothelial derived relaxing factors (nitric oxide, prostacyclin) in control of coronary blood flow during hypoxia. Additionally, reductions in PO_2 hyperpolarize coronary vascular smooth muscle via K^+_{ATP} channels which represent important "end effectors" that significantly contribute to hypoxic coronary vasodilation. Taken together, these data collected over the past 75+ years support the hypothesis that the coronary vascular smooth muscle response to hypoxia depends on metabolic and endothelial vasodilatory factors that are produced in proportion to the degree of hypoxemia that function through mechanisms depending on K^+_{ATP} channels.

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Chapter 4

METABOLIC CONSEQUENCES OF INTERMITTENT HYPOXIA

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Abstract: Insulin resistance is being recognized increasingly as the basis for the constellation of metabolic abnormalities that make up the metabolic syndrome, or Syndrome X. Insulin resistance is also the primary risk factor for the development of type 2 diabetes mellitus, which is currently reaching epidemic proportions by affecting more than 170 million people worldwide. A combination of environmental and genetic factors have led to a dramatic rise in visceral adiposity, the predominant factor causing insulin resistance and type 2 diabetes. Visceral adiposity is also the major risk factor for the development of Sleep Apnea (SA) - an association that has fueled interest in the co-morbidity of SA and the metabolic syndrome, but hampered attempts to ascribe an independent causative role for Sleep Apnea in the development of insulin resistance and type 2 diabetes. Numerous population and clinic-based epidemiologic studies have shown associations, often independent of obesity, between SA (or surrogates such as snoring) and measures of glucose dysregulation or type 2 diabetes. However, treatment of SA with continuous positive airway pressure (CPAP) has not been conclusive in demonstrating improvements in insulin resistance, perhaps due to the overwhelming effects of obesity. Here we show that in lean, otherwise healthy mice that exposure to intermittent hypoxia produced whole-body insulin resistance as determined by the hyperinsulinemic euglycemic clamp and reduced glucose utilization in oxidative muscle fibers, but did not cause a change in hepatic glucose output. Furthermore, the increase in insulin resistance was not affected by blockade of the autonomic nervous system. We conclude that intermittent hypoxia can cause acute insulin resistance in otherwise lean healthy animals, and the response occurs independent of activation of the autonomic nervous system.

Key Words: glucose, mouse, oxidative, sympathetic nerve activity, type 2 diabetes.

INTRODUCTION

Obesity is a major risk factor for the development of metabolic dysfunction. In the United States and other Western countries, obesity is reaching epidemic proportions in adults and its prevalence is rising dramatically in children (9; 24; 26). Obesity, especially central obesity leads to insulin resistance and type 2 DM, affecting more than 100

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million people worldwide. Central obesity is also a major risk factor for sleep apnea (SA), which affects 2-4% of adults (45) and has a prevalence in excess of 50% in obese, otherwise healthy males (32; 46). Thus, both metabolic dysfunction and SA are adverse outcomes of obesity and act as important intermediates in the path to cardiovascular morbidity and mortality.

The fact that SA and diabetes frequently co-exist in obese individuals has received growing attention in the clinical literature (13; 32; 43). Some investigators attributed this coexistence to obesity being a common risk factor for both SA and diabetes (36), whereas others have suggested that there are physiological links between SA and insulin resistance, independent of obesity (13; 32; 38; 39; 43). We recently undertook a systematic review of the literature on the relationship between SA and glucose intolerance and insulin resistance (30). The overwhelming majority of large community and clinic-based studies reported a positive association between SA and one or more parameters of metabolic dysfunction, independent of obesity (1; 6; 7; 8; 10; 13; 17; 22; 32; 39; 41; 43). Moreover, there was evidence of an association between glucose intolerance and insulin resistance and the presence of hypoxic stress in SA patients (13; 31). In contrast, several small interventional studies were unable to demonstrate any significant metabolic improvements with the application of continuous positive airway pressure (CPAP) to abolish SA (2-5; 14; 33; 34; 37). However, a recent study by Harsch et al.(11) conducted serial measurements of insulin sensitivity using the gold standard of the hyperinsulinemic euglycemic clamp in forty SA patients before and after treatment with CPAP. They observed significant improvements in insulin sensitivity with CPAP treatment in apneic patients. Furthermore, they made the interesting observation that the less obese patients had the greatest improvements in insulin sensitivity with CPAP treatment. However, to this point there has been no direct cause and effect evidence linking SA to insulin resistance.

INTERMITTENT HYPOXIA AND INSULIN RESISTANCE

We have recently undertaken a series of studies in mice to examine the effects of intermittent hypoxia (IH), the predominant physiological disturbance in SA, on the dysregulation of glucose homeostasis and the development of insulin resistance. In general the model uses a paradigm of repetitive acute hypoxic exposures in which the inspired oxygen level is reduced from room air levels of 20.9% to 5-6% over a 30 sec period and then reoxygenated back to room air levels over the ensuing 30 sec period (29). The resulting hypoxic exposure of 1 event/min or 60 events/hr is maintained for the 12 hours of the light, or sleeping, phase of the mouse. During the intervening dark, or active phase of the mouse the oxygen environment is maintained at room air levels for the entire 12-hour period. The duration of IH-exposure varies between studies from as short as nine hours to as long as 3 months. We have conducted several studies that address the question of whether exposure to IH has a detrimental impact on glucose regulation and insulin sensitivity.

In an initial series of experiments we determined that five days of exposure to IH

actually lowered blood glucose levels and improved glucose tolerance (28). This apparent increase in insulin sensitivity was observed in both lean C57BL/6J mice and genetically obese ob/ob mice, and was counterintuitive to any hypotheses based on the clinical literature detailed above indicating that the hypoxic stress of SA was associated with insulin resistance. However, the protocol adopted in these initial murine studies assessed blood glucose levels and glucose tolerance at least six hours after the last period of IH exposure. In a subsequent series of studies focusing on the effects of IH on lipid metabolism (18; 21) it became evident that glucose levels were potentially affected by the timing of sacrifice relative to the hypoxic stress. Glucose levels were in fact elevated in C57BL/6J mice when the animals were immediately removed from the hypoxic environment. Thus the concept emerged that insulin resistance may be limited to the period of exposure to IH, and at times of room air exposure during the dark period insulin sensitivity returns to normal or is even potentially improved.

To definitively demonstrate that insulin resistance occurs during the time of exposure to IH we developed techniques to perform the hyperinsulinemic euglycemic clamp in conscious, chronically instrumented, unhandled mice. Catheters were chronically implanted in the femoral artery for assessment of blood glucose and the femoral vein for infusion of insulin, glucose, and saline. The more difficult catheterization of the femoral artery and vein was chosen in preference to the carotid artery and jugular vein to preserve an intact blood flow to the carotid body, the major sensory system detecting hypoxemia. The theoretical basis of the clamp is that if the body is exposed to high levels of insulin then the amount of exogenous glucose required to maintain euglycemia is directly proportional to the whole-body insulin sensitivity: the more exogenous glucose required the greater the insulin sensitivity. We utilized the clamp in a group of lean C57BL/6J mice during the last two hours of a nine-hour exposure period to IH (12). We observed that during IH exposure there was a 21.4% reduction in the level of exogenous glucose infused to maintain euglycemia (Figure 1). These data demonstrate a cause and effect relationship between hypoxic stress and the development of insulin resistance, independent of obesity or any other confounding factors that are common in clinical SA.

Although IH clearly impaired insulin sensitivity, there was no evidence that glucose production by the liver was impacted by hypoxic stress (12). Hepatic glucose output was assessed over an 80-minute period immediately prior to beginning the hyperinsulinemic euglycemic clamp, and there was no statistical difference between animals exposed to IH or intermittent air. However, in contrast to glucose metabolism in the liver, IH significantly impacted on the ability of skeletal muscle to utilize glucose. There were marked reductions in the ability of oxidative muscle fibers to take up glucose during exposure to IH (Figure 2). On the other hand, glucose uptake of glycolytic muscle fibers was unaffected by IH. An intermediate response was found for mixed oxidative/glycolytic muscle fibers; that is, IH did cause reductions in muscle glucose uptake, but the effect was attenuated compared to purely glycolytic muscle fibers. Thus, muscle tissue, the major source of glucose disposal under hyperinsulinemic conditions, is compromised with respect to glucose flux in oxidative fibers during exposure to IH.

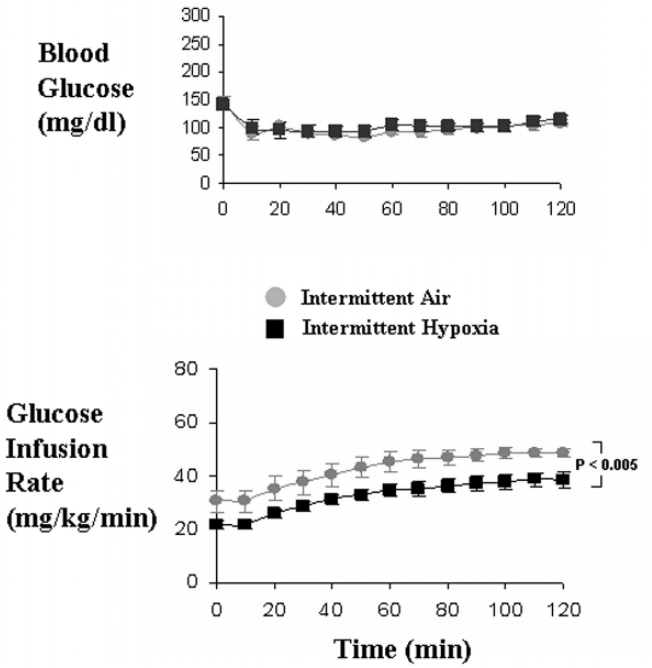

Figure 1. In neurally intact animals, insulin sensitivity was assessed by the steady-state level of exogenous glucose infusion during the 90-120-minute time period under conditions of hyperinsulinemia (20 mU/kg/min) in mice exposed to either intermittent hypoxia or intermittent air (control). Statistical differences between the means of the last 30 minutes of glucose infusion in the intermittent air and intermittent hypoxic groups were determined by unpaired two-tailed t test. Reprinted, with permission, from (12).

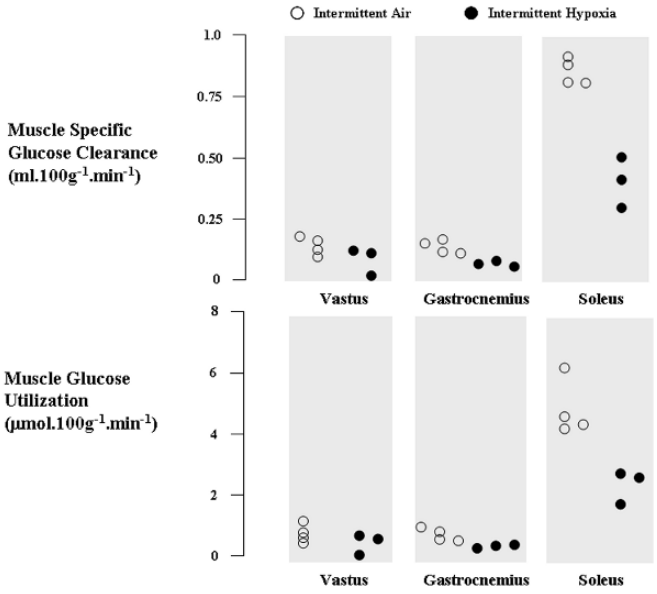

Figure 2. Muscle-specific glucose clearance and muscle glucose utilization in the vastus, gastrocnemius, and soleus muscle were determined in seven mice with intact autonomic nervous systems under intermittent hypoxia and intermittent air conditions. Reprinted, with permission, from (12).

MECHANISMS OF INSULIN RESISTANCE DURING INTERMITTENT HYPOXIA

The data related to reduced glucose uptake in muscle fibers suggest that an intrinsic characteristic of the oxidative muscle fibers contributes to the presence of insulin resistance. It is possible that during IH a reduction in oxygen delivery to muscle effectively slows the rate of oxidative metabolism and decreases the rate of glycolysis. As a result insulin is not as effective at disposing glucose in the oxidative soleus muscle compared to the glycolytic vastus muscle, which is much less dependent on oxidative metabolism. Thus, a reduction in oxygen delivery may contribute to the presence of an "insulin-resistant-like state" during acute IH exposure. However it remains to be seen whether these acute, brief periods of hypoxia are sufficient to interfere with oxidative metabolism. The observation described above, namely that during room air exposure at the completion of 12 IH exposure there may actually be an increase in insulin sensitivity, suggests that a compensatory response to the IH exposure may occur and such a response could not be explained by simply restoring a normal oxygen flux to the metabolizing issues.

Consequently, other factors are likely contributing to the development of insulin resistance during IH exposure. One potential mechanism is the increase in sympathetic nerve activity (SNA) that is an established sequelae of both IH and SA. Increased SNA is a major contributor to both the acute fluctuations in blood pressure that occur in SA patients during acute periods of airway obstruction at night and also for the sustained hypertension that occurs during unobstructed breathing during the daytime (25; 35). Since activation of the sympatho-adrenal axis reduces insulin sensitivity and moblizes glucose in the blood, it has been hypothesized that elevated SNA may contribute to the development of insulin resistance in SA (15; 23; 27). The fact that insulin sensitivity can improve as rapidly as 48 hours after inititiation of CPAP treatment in SA patients has further lead to speculation of a neural mechanism mediating insulin resistance (11). However the role of SNA in modulating insulin sensitivity in response to SA or IH has not been previously tested.

In our chronically instrumented murine model, we examined the impact of SNA on the development of insulin resistance during acute exposure to IH. We hypothesized that blockade of the entire autonomic nervous system, including release of adrenal catecholamines, with the ganglionic blocker hexamethonium would attenuate the insulin resistance that we observed (see above) during exposure to IH. Interestingly, we found that there was no reduction in the magnitude of insulin resistance that occurs during IH with autonomic blockade, suggesting that under the conditions of acute IH exposure in our animal model there was no contribution of SNA in the development of insulin resistance. However, these findings do not rule out the possibility that it in the setting of clinical SA that chronic over-activation of SNA potentially contributes to the development of insulin resistance.

In addition to the sympathetic adrenal axis there are many other potential pathways for the hypoxic stress of SA to impact on insulin sensitivity. A wide variety of mechanisms have been implicated in the development of insulin resistance in obesity that

may also be relevant to IH or SA. One mechanism that has received relatively minor attention in both the obesity and SA literature is the activation of the hypothalamic-pituatary-adrenal (HPA) axis. Cortisol in humans and corticosterone in rodents are important counter-regulatory hormones that reduce insulin sensitivity. We observed in our murine model that IH exposure resulted in elevated cortisol levels, even after blockade of the autonomic nervous system (12). Therefore, activation of the HPA axis may play a role in the development of insulin resistance in response to IH exposure.

More recently a lipotoxicity hypothesis of obesity-related insulin resistance has received attention (40; 42; 44). The lipotoxic effects on insulin sensitivity can occur through elevated circulating cytokines from adipose tissue, ectopic fat deposition, hyperlipidemia, and generation of reactive oxygen species. Many, if not all, of these lipotoxic pathways are potentially activated in obstructive SA although the confounding role of obesity complicates any interpretation. However, data from rodent models of IH demonstrate that hypoxic stress can cause hyperlipidemia, increased deposition of triglycerides in the liver, elevation of circulating cytokines and increased lipid peroxidation (19-21). All of these lipotoxic responses occurred independent of obesity indicating a potential role in the development of insulin resistance in response to hypoxic stress.

SUMMARY

Studies in rodent models of IH demonstrate that insulin resistance occurs during periods of hypoxic stress, independent of the presence of obesity. Surprisingly, the rapid onset of insulin resistance in this rodent model of IH is not dependent on activation of the autonomic nervous system. Rather, other mechanisms including loss of the normal oxygen flux to oxidative muscle fibers, activation of the hypothalamic-pituitary-adrenal axis, or stimulation of lipotoxic pathways could all potentially play a role in IH-induced insulin resistance. If the findings in rodent models of IH translate to the clinical arena it suggests that the hypoxic stress of SA may result directly in metabolic dysfunction leading to the development of insulin resistance and hastening the onset of type 2 diabetes.

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Chapter 5

INTERMITTENT HYPOXIA AND COGNITIVE FUNCTION: IMPLICATIONS FROM CHRONIC ANIMAL MODELS

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Abstract: Obstructive sleep apnea syndrome (OSAS) is a frequent sleep disorder in which the upper airway collapses repeatedly during sleep, resulting in intermittent hypoxia (IH) and asphyxia, and leading also to sleep fragmentation due to the recurrent nocturnal arousals necessary to relieve the upper airway obstruction. In addition to cardiovascular and metabolic morbidities, OSAS also causes serious neurocognitive daytime dysfunction and is associated with regional alterations in brain morphology in humans. These findings suggest that the anatomical brain lesions may underlie the behavioral deficits associated with the disease. In rodents, chronic exposure to intermittent hypoxia (IH) during sleep, which model the hypoxia/re-oxygenation patterns observed in moderate to severe OSAS patients, replicates many of the neurocognitive features of OSAS in humans, such as learning and memory deficits and impaired vigilance. Exposure to experimentally-induced IH in the rodent is also associated with age- and time-related neurodegenerative changes in addition to alterations in brain regions and neurotransmitter systems involved in learning and memory, attention, and locomotor activity. Multiple pathophysiological processes appear to be involved in the mechanistic aspects of the behavioral and neuronal susceptibility to IH during sleep, and include pathways leading to increased oxidative stress and inflammation, altered gene regulation, and decreases in the cellular and molecular substrates of synaptic plasticity. In addition, both environmental and genetic factors modulate the end-organ susceptibility to IH-induced cognitive dysfunction in rodents. Collectively, the available data indicate that exposure to IH during sleep is associated with adverse behavioral and neuronal consequences in the rodent. Improved understanding of the determinants of IH-related susceptibility may help explain the phenotypic variance in OSAS-associated morbidities, and enable improved therapeutic approaches in the future.

Key Words: sleep apnea, oxidative stress, inflammation, learning

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OBSTRUCTIVE SLEEP APNEA SYNDROME (OSAS)

Obstructive sleep apnea syndrome (OSAS) is a frequent sleep disorder in which the upper airway collapses repeatedly during sleep, resulting in intermittent hypoxia (IH), asphyxia, and associated sleep fragmentation due to the recurrent nocturnal arousals necessary to relieve the upper airway obstruction. The hypoxic pattern most often observed in patients with OSAS consists of apneic episodes of relatively short duration interspersed between normoxic periods that last several minutes, and typically recur throughout the sleep cycle. The constellation of neuronal, cardiovascular, and metabolic pathologies now known to be associated with OSAS has intensified interest in the long-term consequences of exposure to such episodic hypoxic profiles, particularly as they relate to neuropathology (16, 80, 102). Impaired cognition, excessive daytime sleepiness, and mood disturbances are typically observed in adult patients with sleep apnea (16, 44, 82). In addition, increased neurodegenerative changes and enhanced susceptibility to oxidative injury has been postulated as a likely consequence of the intermittent hypoxia associated with OSAS (66), a prediction that has been borne out by a number of neuroimaging studies reporting that adult patients who suffer from OSAS develop regional gray and white matter losses and display alterations in markers of neuronal integrity, as well as show changes in prefrontal lobe perfusion (2, 6, 25, 45, 46, 57, 94). Although not all studies have replicated these findings (72), the majority of such imaging studies support the existence of neurodegenerative processes in patients with OSAS (67). The loss of gray matter that occurs in the hippocampal and parahippocampal brain regions of OSAS patients (63) lends further credence to the hypothesis that disruption of neuroanatomical integrity in brain regions involved in learning and memory is a consequence and underlying factor in OSAS-associated neurocognitive morbidity. Although such studies do not allow separation of the hypoxemia associated with OSAS from additional features of the disease, such as hypercapnia and sleep fragmentation, it is clear that knowledge of the effects of chronic cyclical hypoxia alone is important for evaluating the impact of specific components of the disease in the overall clinical picture of patients with sleep apnea.

INTERMITTENT HYPOXIA

Intermittent hypoxia (IH) is traditionally defined as the episodic occurrence of hypoxia, interspersed between normoxic periods with subsequent reoxygenation. This definition obviously encompasses a wide variety of hypoxic exposures, which can exert both adaptive and maladaptive effects, depending on both the manner of presentation of the hypoxic stimulus as well as the target tissue examined (15, 32, 66, 90). The degree of chronicity of the IH presentation has been postulated to trigger the shift towards pathological consequences (66). Given that even within an experimental paradigm, factors such as the frequency and duration of the hypoxia-reoxygenation cycles, variations in severity, as well as the overall time course of exposure can have a dramatic impact on the response to IH (60, 80), this mini-review will focus primarily on recent

experimental findings in animal models of the chronic hypoxia-reoxygenation events that occur in patients with obstructive sleep apnea syndrome (OSAS).

COGNITIVE AND BEHAVIORAL EFFECTS OF IH

The patterns of cognitive disturbances seen in OSAS patients are consistent with dysfunction of fronto-subcortical systems involved in attention and executive functions, such as the dorsal prefrontal (PFC) cortex, as well as regions involved in the consolidation of learning and memory, such as the hippocampus (2, 5, 9, 10, 22, 23, 30, 65, 71, 103). Growing awareness of the potential contribution of episodic hypoxia to such neurocognitive consequences has led to the use of animal models that replicate the breathing patterns of patients with OSAS to investigate links between such exposures and cognitive function and behavior. Chronic exposure to IH, in the absence of significant sleep disruption, has been shown to produce deficits on spatial learning tasks in the rat (32). Subsequent studies have found both rats and mice to display cognitive deficits consistent with impaired functioning of the hippocampus and/or prefrontal cortex following exposure to IH (17, 34, 86, 88, 89). Experimental models have also implicated IH as an underlying factor in hypersomnolence, as exposure to IH during the rodent sleep cycle has been shown to result in impaired maintenance of wakefulness in mice, suggesting that such exposures are an underlying contributor to the excessive daytime sleepiness observed in patients with OSAS (101). Collectively, these findings illustrate that exposure to IH is capable of replicating many of the cardinal neurocognitive morbidities associated with OSAS, and provide further support for the hypothesis that chronic exposure to IH is an important component of cognitive and behavioral dysfunction seen in patients with sleep apnea.

Behavioral problems, such as an increased incidence of hyperactivity and behavioral disturbances reminiscent of the hyperactive/impulsive subtype of attention-deficit hyperactivity disorder (ADHD), are frequently reported in children with OSAS and related forms of sleep-disordered breathing (40) . Exposure to IH at an age period that ontogenetically corresponds to the age of peak incidence of OSAS in children (31, 33) has been shown to enhance locomotor activity in the open field of male, but not female, rats (88). Similar non-gender dependent enhancement of locomotor activity has been observed in rats exposed to intermittent hypoxia at an earlier developmental period that corresponds more closely with the preterm human born between 32 and 36 weeks of age (17, 18). Given that even mild chronic and intermittent hypoxia in childhood is associated with alterations in behavior and cognitive performance (7), it suggests that intermittent hypoxic exposure during critical developmental periods is a contributing factor to the behavioral pathologies seen in pediatric OSAS.

Exposure to IH also appears to have long-term behavioral consequences in both the adult and developing animal. Adult males exposed to IH show only partial recovery of learning after two weeks of recovery (32). Deficiencies in spatial working memory were found in male, but not female rats, exposed to IH from post-natal days 10-25, when tested four months after the cessation of the hypoxic exposures (49). Additionally, juvenile rats, exposed to IH from post-natal days 7-11, display working memory

impairments when tested at 65 days of age (17). Furthermore, exposure to intermittent hypobaric hypoxia from birth until the age of 19 days was found to impair spatial learning as much as seven months after termination of IH (93). This suggests that the behavioral consequences of IH may be long-lasting and only partially reversible, although it is at present unclear the degree to which these changes are due to direct alteration in hippocampal and cortical regions, or represent dysregulation of related sub-cortical structures brought about by compensatory responses to IH.

NEURONAL PATHOPHYSIOLOGY OF CHRONIC IH

Severe hypoxia is well established as a major pathological factor capable of inducing impairment of brain function and neuronal cell injury that ultimately results in neurodegeneration and cell death (69). Although our existing knowledge of the effects of mild, episodic hypoxia is less complete, recent animal studies are beginning to shed light on the relationship between the behavioral and neuronal consequences of such exposures. Gozal et. al. (2001) initially demonstrated that exposure to IH is associated with increases in programmed cell death, or apoptosis, as well as cytoarchitectural disorganization in brain regions involved in learning and memory, such as the hippocampal CA1 region and the frontoparietal cortex, while evoking virtually no changes in more resistant brain regions, such as the CA3 region of hippocampus and the dorsocaudal brainstem (32). Proteomic approaches have subsequently shown that substantial differences in protein regulation, primarily related to structural, apoptotic, and metabolic pathways occur between IH sensitive and IH resistant brain regions (36). In addition, enhanced glial proliferation occurs in the rat cortex following IH exposure (32), possibly indicating a pathological activation of microglia, a common feature of neurodegeneration (56, 107). In mice, neurodegenerative changes have also been reported in sleep-wake regions of the brain, such as the basal forebrain, following more chronic exposures (101). More recently, reductions in N-acetyl aspartate/creatine (NAA/Cr) ratios, a non-invasive marker of neuronal integrity, have been demonstrated in mice exposed to 4 weeks of an intermittent hypoxia profile (19).

These changes are paralleled by IH-induced alterations in the cellular and molecular substrates of synaptic plasticity within the hippocampus and cortex. For example, IH has been demonstrated to diminish the ability of hippocampal neurons to sustain long term potentiation (LTP), a physiological NMDA-dependent mechanism thought to be a biological correlate of learning and memory (78), as well as decrease excitability of hippocampal CA1 neurons (39). Furthermore, IH is associated with reduction of the density of NMDA R1 receptor expressing cells in the CA1 field of hippocampus (32), suggesting the involvement of exitotoxic processes that may contribute to NMDA expressing cells being especially vulnerable to IH (1, 32). At the cellular level, IH has been demonstrated to induce significant decreases in the Ser-133 phosphorylation of the cAMP-response element binding protein (pCREB), a transcription factor that mediates critical components of neuronal survival and memory consolidation in mammals within the IH-sensitive hippocampal CA1 region. Decreases in CREB phosphorylation, without changes in total CREB, were found to peak between 6 hours and 3 days of

exposure, and gradually returned toward normoxic levels by 14-30 days (28). IH was also found to induce a similar pattern of time-dependent biphasic changes in downstream anti-apoptotic pathways within the CA1 region, such as protein kinase B (AKT) and glycogen synthase kinase 3beta (GSK-3ß), which were temporally correlated with alterations in IH-induced neuronal apoptosis (27).

Experimental studies in rats have established the existence of a developmental period ranging from 10 to 25 days after birth, during which IH exposure results in a dramatic increase in number of cortical and hippocampal neurons undergoing apoptosis in comparison to both neonatal and adult rats (37). Such findings are consistent with previous work demonstrating that the brain is particularly vulnerable to hypoxia during periods of maturation and development, and that hypoxic episodes occurring during these critical periods seriously impact brain maturation, with consequences ranging from cell death to impaired differentiation of dendrites and axons, and to decreases in synapse formation (68-70). Severe perinatal and postnatal forms of hypoxia/ischemia or prolonged anoxia are associated with cognitive and motor impairments (20, 38, 99). Similar developmental windows of susceptibility have been demonstrated in rodent models that employed significantly more severe hypoxia or anoxia (24, 98, 100) suggesting the effects of IH exposure in the immature animal may involve additive or synergistic effects between ongoing developmental apoptosis and the hypoxic stimulus, which may account for the alteration in behavioral phenotype seen after early post-natal IH exposure (86).

Intermittent hypoxia during such early periods of vulnerability may also result in long-term effects due to disruption of brain growth and maturation, ultimately leading to long-term neurocognitive morbidity (17, 18, 32, 49, 88). Several reports now indicate that IH exposure is capable of producing long-term neuroantomical consequences. Early post-natal exposure to IH is associated with disorganization of the processes of astroglial and oligodendroglial cells in the cortex and hippocampus (93), as well as decreased dendritic arborization in the prefrontal cortex of male, but not female, rats 4 months post-hypoxia, in addition to non-gender dependent alteration of aminergic pathways (49). In mice, irreversible decreases in myelination of the corpus callosum, which may ultimately compromise connectivity between cortical regions, have been observed after 4 weeks of IH exposure (47). These findings, when taken in conjunction with behavioral studies, indicate that IH may not only have an acute impact on brain function, but may also modify the development of neural networks underlying such higher-order functions such as attention, as well as learning and memory.

Patients suffering from OSAS have increased circulating markers of oxidative stress and inflammation, suggesting that oxidative injury and inflammation may be an underlying cause of OSAS-associated morbidities (14, 51, 104). In experimental models, increased inflammation and oxidative stress have been implicated as a crucial factor in the adverse neurobehavioral consequences of IH, since anti-oxidant treatment will ameliorate IH-induced learning deficits in rats, as well as IH-induced hypersomnolence in mice (89, 114). In addition, transgenic mice overexpressing the anti-oxidant enzyme Cu,Zn-superoxide dismutase display a lower level of overall ROS production, as well as reductions in cortical neuronal apoptosis in comparison to normal control mice when exposed to IH, which further suggests a causal role for oxidative stress in IH-

mediated neuronal apoptosis (111). Intermittent hypoxia-induced spatial deficits and apoptosis are more severe in aged rats, an effect thought to be partly due to a reduction in anti-oxidant capabilities and subsequent adverse downstream effects on ubiquitin-proteasomal pathways, (35, 42). Increases in lipid peroxidation, carbonylation and nitrosylation-induced oxidative injury have been demonstrated in sensitive brain regions of both rats and mice following chronic exposure to IH (89, 91, 101, 113). Upregulation of the inflammatory iNOS and cyclooxygenase-2 (COX-2) pathways is seen in hippocampal and cortical regions of the rat brain after exposure to IH, and have been linked to the neurobehavioral deficits seen after such exposures (54, 55). Furthermore, absence of the platelet-activating factor (PAF) receptor, an important bioactive mediator of oxidative stress and inflammation, confers protection against IH-induced neurobehavioural deficits and attenuates increases in iNOS and COX-2, implicating PAF in the inflammatory signalling pathways induced by IH (87). Such increases of oxidative and inflammatory processes may have important implications, especially considering the observed IH-induced increases in glial proliferation (32). Astroglial and microglial cells play central roles in inflammatory and oxidative processes in the brain, as activated astroglial and microglial cells express high levels of iNOS and cyclooxygenase-2 (COX-2), which are capable of initiating pathways ultimately leading to production of a variety of reactive oxygen species that have been implicated in experimental models of neurodegeneration and ischemia/reperfusion injury (21). In turn, cytokine secretion facilitates production of ROS, as well as iNOS and COX-2 by microglia, thus perpetuating inflammation and aggravating ongoing oxidative stress (41, 113). Consistent with these findings, both pharmacological and genetic inhibition of iNOS confers resistance to IH-induced learning deficits and hypersomnolence (55, 113). Finally, recent work suggests that upstream activation of NADPH oxidase, a major contributor to oxidative injury in the brain and other target organs under conditions of inflammation and severe hypoxia (52, 64, 74, 108, 110), may underlie the adverse neuronal consequences of IH, since both genetic and pharmacologic inhibition of this enzyme is capable of abrogating the inflammatory and oxidative cascades observed in animal models of IH (114). Table 1 summarizes the mechanisms implied in the adverse neuronal consequences of IH exposure.

The available evidence therefore indicates that the pathophysiological consequences of IH are due to a complex interplay of both oxidative and pro-inflammatory responses which initiate patterns of cellular activation ultimately leading to dysfunction within sensitive brain regions (illustrated in Figure 1).

Table 1. Rodent studies implicating mechanistic pathways in the adverse neuronal consequences of intermittent hypoxia (IH) in rodents (Cox-2: cyclooxygenase 2; iNOS: inducible nitric oxide synthase; PAF: platelet activating factor).....

Mechanism	Brain Regions Examined	Citation
Apoptosis	•Hippocampus (CA1) •Cortex	• 28, 32, 35, 37, 54, 87, 111
Lipid Peroxidation	•Hippocampus •Cortex •Basal Forebrain	• 50, 89, 91,101, 111, 113, 114
iNOS	•Hippocampus •Cortex •Basal Forebrain	• 50, 54, 87, 91, 101, 113
COX-2	•Hippocampus •Cortex •Basal Forebrain	• 50, 55, 87
Nitrosylation/ Carbonylation	•Hippocampus •Cortex •Basal Forebrain	• 91, 101, 113, 114
PAF	•Hippocampus •Cortex	• 87
Altered gene regulation	•Hippocampus •Cortex •Basal Forebrain	• 17, 18, 27, 28, 29, 36, 85, 86, 101, 111, 113, 114

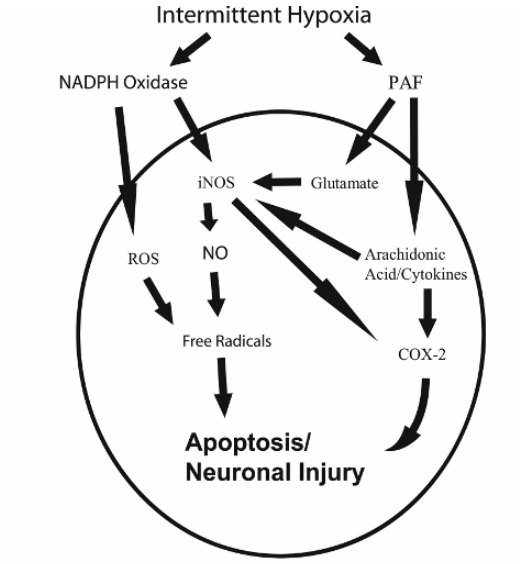

Figure 1. Putative model of the mechanisms implicated in the adverse neuronal consequences of intermittent hypoxia (IH) in rodents. IH elicits excessive activation of oxidative and inflammatory pathways which are capable of inducing cellular damage, in addition to initiating pathological cross-talk and activation of positive feedback loops which contribute to cellular dysfunction within susceptible neurons (Cox-2: cyclooxygenase 2; iNOS: inducible nitric oxide synthase; PAF: platelet activating factor; ROS: reactive oxygen species).

Alterations in neurotransmitter systems have also been implicated in the neurobehavioral disturbances seen after IH exposure. Several studies have shown dysregulation of dopaminergic and catecholaminergic systems following IH exposure, particularly in developing animals. Li et. al. exposed rats to 8 hours daily of varying IH profiles and found that episodic hypocapnic hypoxia produced a decrease in dopamine turnover while eucapnic hypoxia increased norepinephrine levels in the hypothalamus, suggesting that episodic eucapnic and hypocapnic hypoxia may affect metabolism of these neurotransmitters in the CNS (53). Decker et. al. found enhanced expression of vesicular monamine transporter (VMAT) and D1 dopamine receptors in the striatum of post-hypoxic (exposed from days 7-11) rats at 80 days of age (17). Furthermore, reduced levels of extracellular dopamine were found in the striatum of these post-hypoxic rats (18). Kheirandish et. al. found related alterations in frontocortical catecholaminergic pathways resulting from exposure to IH during a critical post-natal developmental period (post-natal days 10-25), which have been postulated to contribute to gender differences in recovery from IH-induced neurobehavioral deficits (49). Such changes in aminergic pathways likely have important implications for the behavioral phenotype associated with IH exposure, as experimental and clinical studies have implicate disruption of dopaminergic pathways in the development of hyperactivity and working memory dysfunction characteristic of disorders of minimal brain dysfunction, such as ADHD (12). For example, dopamine D1 receptors play an important role in working memory mechanisms across species (92, 109), whereas polymorphisms in the dopamine D4 receptor gene in children may contribute to behavioral problems characterized by impulsive and hyperactive behavior (26). Furthermore, D4 receptors appears to be essential for hyperactivity and impaired behavioral inhibition in rodents (59, 105). Expression of dopamine and modulation of its receptors are subject to significant developmental alteration which can be readily influenced during critical periods of development by factors such as gender and external stress (3, 8, 58). Dysregulation of dopaminergic function, rather than absolute levels of dopamine, appears to be the important determining factor for behavioral alterations, as both hypodopaminergic and hyperdopaminergic animal models have been implicated in behavioral and cognitive dysfunction (4, 106). These findings clearly suggest that alterations and disruption of tyrosine hydroxylase (TH)-related pathways underlie, or at least contribute, to the adverse functional outcomes associated with IH.

Although the available data indicate that alterations in catecholaminergic, and in particular dopaminergic, systems play a central role in the behavioral pathology associated with IH exposure, it should also be noted that the influences of other neurotransmitters will need to be considered, especially given the established role of serotonin in ventilatory plasticity in response to IH (11, 82, 84, 86, 102). Furthermore, exposure to IH results in increased nitrosylation and carbonylation in sleep-wake regions of the brain, such as the basal forebrain. In addition to the established role of this region in the hypersomnolence and increased susceptibility to short-term sleep loss observed in mice exposed to IH (91, 101, 113, 114), these findings also have important implications for cognitive function, as the basal forebrain also provides cholinergic innervation of hippocampal and cortical structures involved in learning and memory (61), suggesting that such changes may also contribute to learning and memory impairments. Recently,

animals exposed to 2 weeks of IH were found to have reduced expression of choline acetyltransferase (CHAT)-positive neurons in the medial septal nucleus, in both the vertical and horizontal limbs of the diagonal band, and the substantia inominata after 14 days of IH exposure, suggesting that a loss of cholinergic neuronal phenotype within the basal forebrain may play a role in the cognitive impairments associated with IH exposure, especially as they pertain to working memory (86).

GENETIC AND ENVIRONMENTAL MODIFICATIONS OF IH-INDUCED COGNITIVE SUSCEPTIBILITY

Like many clinically complex disorders, OSAS has a multifactorial etiology that is currently presumed to involve a number of candidate genes that may predispose individuals to risk factors associated with the disease, such as obesity/central apidposity and regulation of upper airway musculature, anatomy, and function (75, 76, 95). However, unlike single gene disorders, which may exert profound effects on the phenotype of the disease irregardless of environmental influences, it is more likely that OSAS-related morbidities results from a complex interplay between multiple genetic influences and environmental interactions within the context of the underlying features of the disorder. A number of environmental sources (such as exposure to alcohol, sedatives, and smoking) been linked to exacerbation of OSAS severity (77, 78), suggesting that such factors, in conjunction with genetic risk factors, may potentially influence the behavioral and neuronal responses to specific components of the disease, such as IH (see figure 2). Recent evidence from animal models now suggests that both genetic and environmental factors are capable of modulating susceptibility to IH. For example, mice lacking the lipid processing gene, ApoE, a well-known risk factor for Alzheimer's disease as well as OSA (13, 43, 73), show increased susceptibility to IH-induced spatial learning deficits in the water maze, as well as increased neuronal inflammation and oxidative stress (50). Similar increases in cognitive vulnerability, have been shown in animals exposed to the environmental component of obesity, a long-term diet high in fat and refined carbohydrates (29). Conversely, positive environmental factors, such as increases in physical activity and environmental enrichment, may be capable of promoting tolerance to the adverse behavioral consequences of IH exposure (85). These findings are not surprising, given the well-established roles that both genes and environment play in maintaining neural function under both pathological and normal conditions (48, 62, 94, 97, 112). Furthermore, these findings illustrate the need to consider role of both genetic and environmental modifiers of IH susceptibility, in addition to overall severity and of exposure, on end-organ dysfunction in both clinical contexts and animal models.

SUMMARY

Chronic exposure to intermittent hypoxia (IH) in rodents, modeling the hypoxia/reoxygenation patterns observed in severe sleep apnea patients, replicates many of the pathological features of OSA in humans, such as neurodegeneration and cognitive deficits. Additionally, exposure to chronic periods of IH is associated with impaired functioning of hippocampal, prefrontal cortical, as well as related subcortical structures that involves complex destructive mechanisms such as free radical generation, generation of inflammatory mediators, altered regulation of cell survival pathways, and excitotoxicity. The existence of a unique period of susceptibility in the developing animal indicates that exposure to intermittent hypoxic insults may have important consequences in the development of behavioral pathology, particularly with regards to the function and maturation of brain systems involved in attention and motor behavior. Episodic hypoxia paradigms, both in isolation and in conjunction with the additional clinical features of OSA, such as hypercapnia and sleep fragmentation, should therefore be useful for the elucidation of specific mechanisms underlying OSA-associated morbidities. Although substantial strides have been made in our understanding of the effect of chronic IH on neuronal function, future studies will need to evaluate the potential impact of both genetic and environmental factors, in addition to further elaborating the myriad of systemic, cellular, and molecular responses observed in response to chronic IH.

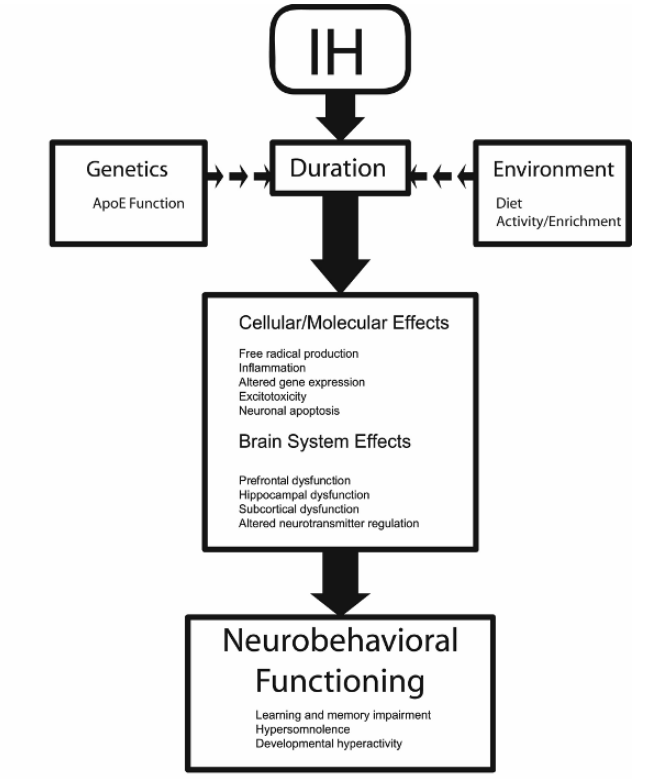

Figure 2. Schematic diagram of the potential interaction of genetic and environmental factors on the cellular and function consequences of intermittent hypoxia (IH) exposure in rodents (ApoE; apolipoprotein E).

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Chapter 6

VASCULAR CONSEQUENCES OF INTERMITTENT HYPOXIA

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Abstract: In patients with obstructive sleep apnea (OSA), nocturnal exposure to intermittent hypoxia causes elevations in arterial pressure that persist throughout the day. Animal models have shown that this hypertensive effect requires an intact sympathetic nervous system and an intact carotid chemoreceptor reflex. The renin-angiotensin system contributes importantly to hypertension in this model, because renal nerve denervation, angiotensin II receptor blockade, and suppression of the renin-angiotensin system by high salt diet all prevent the rise in blood pressure. The vascular endothelium is functionally impaired in this model and also in patients with OSA. These individuals demonstrate decreased plasma levels of nitric oxide metabolites, increased production of superoxide by neutrophils, and increased levels of 8-isoprostane in breath condensate. Increased levels of pro-inflammatory cytokines are also present. Thus, oxidant stress and inflammation are potential mediators of intermittent hypoxia-induced vascular dysfunction. Once the mechanisms of intermittent hypoxia-induced alterations in vascular structure and function are understood, strategies can be developed to reverse or prevent them. Such research has relevance not only to hypertension, but also to atherosclerosis and other important cardiovascular sequelae of OSA.

Key Words: sleep apnea, sympathetic nervous system, chemoreceptor reflex, vascular reactivity

INTRODUCTION

Obstructive sleep apnea (OSA) is associated with hypertension and other forms of cardiovascular morbidity. OSA-related hypertension is characterized by sympathetic nervous system overactivity, impaired endothelial function, and vascular remodeling. Episodes of OSA impose multiple insults; however, intermittent hypoxia, rather than hypercapnia, sleep disruptions, or intrathoracic pressure oscillations, is thought to be the most important pro-hypertensive factor. While much is known about the acute vascular effects of hypoxia, the mechanisms by which acute exposures lead to long-term adaptations in vascular regulation are just beginning to be elucidated.

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EFFECTS OF ACUTE HYPOXIC EXPOSURE ON VASCULAR REGULATION

The caliber of arterioles, the resistance vessels in the systemic circulation, is determined by the net effect of multiple constrictor and dilator influences (Figure 1), many of which are altered by hypoxia. Acute exposure to hypoxia produces a generalized, dose-dependent increase in sympathetic vasoconstrictor outflow caused primarily by engagement of the carotid chemoreceptor reflex (2; 13; 88). Evidence for this effect in humans is a brisk, hypoxia-induced increase in muscle sympathetic nerve activity (80; 81). Hypoxia is largely responsible for the dramatic rise in sympathetic outflow to skeletal muscle during episodes of OSA and during voluntary breath-holds (56; 62).

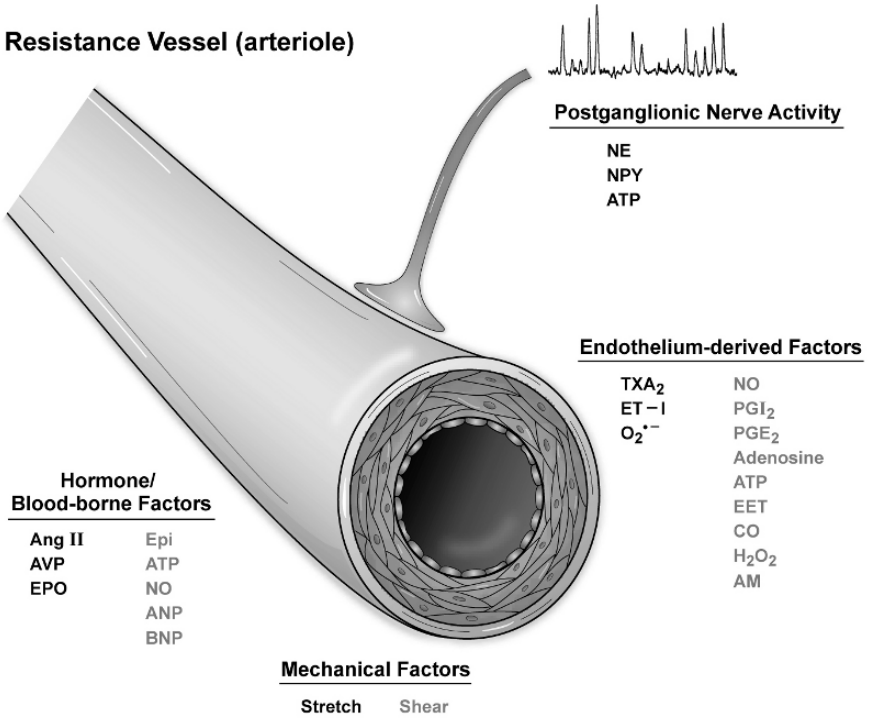

Figure 1. Summary of the neural, chemical, and mechanical factors that determine the caliber of resistance arterioles in the skeletal muscle circulation. Constrictor influences (shown in black) are opposed by dilator influences (shown in gray). Ang II, angiotensin II; AVP, arginine vasopressin, Epi, epinephrine; ATP, adenosine triphosphate; ANP, atrial natriuretic peptide, BNP, brain natriuretic peptide; NE, norepinephrine, NPY, neuropeptide Y; TXA2, thromboxane; ET-I, endothelin-I; O2•–, superoxide; NO, nitric oxide; PGI2, prostacyclin; PGE2, prostaglandin E2; EET, eicosatrienoic acid, CO, carbon monoxide; H2O2, hydrogen peroxide; AM, adrenomedullin.

Acute hypoxic exposure also alters blood-borne regulators of resistance vessel tone. Circulating levels of the constrictor substances angiotensin II (Ang II) and endothelin-I are increased by hypoxic exposure (33; 95). Plasma from hypoxia-exposed rats caused

vasoconstriction in cremaster arterioles of normoxic animals, an effect that was abolished by blockade of Ang II receptors or inhibition of angiotensin converting enzyme (35).

During acute hypoxic exposure, these constrictor influences are opposed by even more powerful vasodilator influences in most vascular beds (the most notable exception is the pulmonary circulation). Peripheral vasodilation during acute hypoxia is caused, in part, by increases in hormones such as epinephrine (96) and atrial natriuretic peptide (54). The erythrocyte is another source of vasodilator chemicals during hypoxic exposure. When hemoglobin becomes deoxygenated, red cells release both nitric oxide (NO) and ATP, which causes vasodilation by binding to purinergic receptors (32).

Endothelium-derived vasodilator substances play crucial roles in hypoxic vasodilation. In the skeletal muscle circulation local hypoxia elicits hyperpolarization of vascular smooth muscle via several distinct pathways. In the predominant pathway, endothelial cells release prostacyclin (27), which binds to a membrane-bound receptor on the vascular smooth muscle cell and activates adenylyl cyclase. Subsequent increases in cAMP lead to the opening of K_{ATP} channels. Another pathway involves endothelial release of NO, stimulation of guanylyl cyclase in the smooth muscle cell, increases in cGMP concentrations, and opening of K_{Ca} channels (28). A third pathway involves reductions in 20-hydroxyeicosatetrienoic acid (20-HETE) levels within vascular smooth muscle cells, which causes further opening of K_{Ca} channels (28). In addition, there is some evidence that endothelial release of epoxyeicosatrienoic acid (EET) contributes to hypoxic vasodilation (29). Thus, hypoxic vasodilation in skeletal muscle occurs through multiple, redundant pathways, and it appears that the specific mechanism called into play in a given situation depends on the severity of hypoxia (28). It is also apparent that during hypoxic exposure endothelial cells release adenosine, which acts in autocrine fashion to cause the release of prostacyclin and NO (78).

Because the balance between constrictor and dilator influences is tipped toward vasodilation in most vascular beds, acute hypoxia has only a modest effect (in some cases no effect) on systemic arterial pressure (91; 102). In rats, arterial pressure decreases upon acute exposure to hypoxia (35; 42) and also during experimentally induced apnea (89). This hypoxic depressor response, which is opposed by chemoreflex-induced increases in sympathetic vasoconstrictor outflow, is mediated by NO (87). In contrast to these acute effects, longer-term hypoxic exposure raises blood pressure in rats and humans (24; 100).

TIME-DEPENDENT NEUROCIRCULATORY ADAPTATIONS TO BRIEF, REPETITIVE HYPOXIC EXPOSURES

Hypertension is highly prevalent in patients with OSA, with estimates ranging from 50-90% (98). Longitudinal data from the Wisconsin Sleep Cohort Study provide epidemiologic evidence for a causal relationship between OSA and hypertension (70). In more than 700 subjects who were normotensive upon entry into the study, the odds ratio for having hypertension at 4-year follow-up was directly correlated with severity of

sleep disordered breathing. Importantly, this analysis controlled for obvious confounding factors such as age, body mass index, smoking, alcohol consumption, and gender. Moreover, a recent prospective study of patients with drug-resistant hypertension (i.e. those whose blood pressures remained elevated in spite of treatment) revealed that the vast majority of these patients had OSA (58).

In animal models of OSA, exposure to intermittent hypoxia or intermittent airway occlusion for 8-14 hours per day produces blood pressure elevations that are evident, not only during the exposure period, but also when the animals are unperturbed and blood gases are normal (5; 24). The model developed by Fletcher and colleagues, which exposes rats to cyclical hypoxia for 8 hours per day during their light cycle, has begun to elucidate the mechanisms responsible for this blood pressure-raising effect (24). In this model, the rise in blood pressure is critically dependent on an intact sympathetic nervous system and an intact carotid chemoreceptor reflex (23; 55). The renin-angiotensin system contributes importantly to hypertension in this model, because renal nerve denervation, Ang II receptor blockade, and suppression of the renin-angiotensin system by high salt diet all prevent the rise in blood pressure (22; 25). Increased blood pressure accompanied by increased endothelin-I levels were observed in rats exposed to intermittent asphyxia (combined hypoxia and hypercapnia) (46). In this model, infusion of a non-selective endothelin receptor antagonist lowered blood pressure in asphyxia-exposed but not control rats. Although the precise mechanisms by which intermittent hypoxia raises blood pressure are unknown, evidence from animal models and from humans with OSA suggests that enhanced sympathetic vasoconstrictor outflow, impairment in vascular endothelial function and vascular remodeling are important contributors (see below).

Intermittent hypoxia-induced sympathetic activation.

Sympathetic nervous system activity is heightened in patients with OSA, both during sleep, when apneic episodes produce intermittent asphyxia and also during wakefulness, when blood gases are normal (6; 39; 64). The mechanisms that maintain sympathetic outflow at high levels after withdrawal of chemical stimuli are incompletely understood; however, the available evidence suggests that the sustained sympathetic activation caused by repetitive exposure to hypoxia has both reflex and central nervous system origins.

In previous human studies from our laboratory, relatively brief (20-minute) exposures to continuous or intermittent asphyxia during wakefulness caused an increase in muscle sympathetic nerve activity that persisted after re-establishment of normoxic, normocapnic conditions (61; 101). Cutler and colleagues found that 20 minutes of repetitive, voluntary breath-holds also produced sustained sympathetic activation (12). Subsequently, we found that the long-lasting sympathoexcitation caused by brief exposure to asphyxia is critically dependent on hypoxia (102).

In rat models, exposure to intermittent hypoxia increased basal sympathetic outflow and resulted in enhanced sympathetic activation during subsequent acute hypoxic exposures (13; 36; 77; 84). Moreover, intermittent hypoxia enhanced carotid body sensory activity, as evidenced by increased rates of carotid sinus nerve discharge during normoxia and upon re-exposure to hypoxia (68; 97). These findings of augmented

carotid chemoreflex function are consistent with human data showing that breathing 100% oxygen reduced sympathetic outflow in patients with OSA but not in matched control subjects (64).

Several recent studies probed the causes of these alterations in chemoreflex function, and much of the existing evidence points to a role for Ang II, endothelin-I and oxidant stress. Infusion of Ang II into the isolated carotid body increased carotid sinus nerve activity (52) and exposure to hypoxia upregulated Ang II type 1 receptors in the carotid body (51). Intermittent hypoxia-induced sensory long-term facilitation of the carotid body was prevented in animals pretreated with a superoxide anion scavenger (68). Administration of a superoxide scavenger also prevented intermittent hypoxia-induced long-term facilitation of respiratory motor output (69). The source of reactive oxygen species in this model appears to be the mitochondrion (50; 76). Endothelin, which is present in glomus cells of the carotid body, produces chemoexcitation by binding to endothelin A receptors (10; 75). The expression of these receptors and of preproendothelin in the carotid body was upregulated by exposure to hypoxia (11). In contrast, NO inhibits carotid body chemosensitivity (88). Two isoforms of NO synthase, eNOS and nNOS, are present in the carotid body (75) and some (97) but not all (3) previous investigators have observed downregulation of these enzymes after exposure to hypoxia. At this point, the role played by NO in causing hypoxia-induced alterations in reflex sympathoexcitation remains unclear.

In addition to its effects on reflex control of sympathetic nervous system activity, exposure chronic intermittent hypoxia may augment central sympathetic outflow. Again, Ang II is a likely contributor to this process. Sympathetic premotor neurons in the brainstem, which are important modulators of postganglionic sympathetic discharge, receive excitatory inputs from higher centers such as the paraventricular nucleus of the hypothalamus and the circumventricular organs. Angiotensin II-containing neurons are located in these regions and, in addition, their weak or absent blood brain barrier allows access to circulating Ang II. Angiotensin II-induced sympathoexcitation is thought to involve NADPH oxidase-derived superoxide ion (106). In its role as a regulator of central sympathetic outflow, Ang II has important interactions with NO (57; 107). Preliminary data from the Weiss lab indicates that exposure to intermittent hypoxia decreases nNOS expression in the paraventricular nucleus and increases angiotensin AT_1 receptor expression in the circumventricular organs (97).

Alterations in vascular function.

Several lines of evidence indicate that the vascular endothelium is functionally impaired in patients with OSA. Reductions in endothelium-dependent vasodilation have been demonstrated using intra-arterial infusion of vasoactive agents (47; 48) and using arterial occlusion to produce flow-mediated dilation in the brachial artery (65). In both cases, indicators of nocturnal hypoxemia (e.g. minimum arterial oxygen saturation, amount of time with saturation <90%) better predicted the degree of endothelial dysfunction than did the frequency of apneas. Evidence for a causal relationship between OSA and endothelial dysfunction comes from a study in which flow-mediated dilation was improved by treatment of with nasal continuous positive airway pressure (CPAP)

(44). This beneficial effect was lost when CPAP was temporarily withheld (Figure 2). More recently, CPAP treatment enhanced the forearm vasodilation produced by intra-arterial infusion of acetylcholine (53). In this study, a greater L-NMMA-induced reduction in forearm blood flow was observed after CPAP, which suggests that elimination of intermittent hypoxia augmented resting NO production. Other investigators found that plasma levels of NO derivatives were suppressed in patients with OSA vs. control subjects and that the levels normalized following CPAP treatment (43).

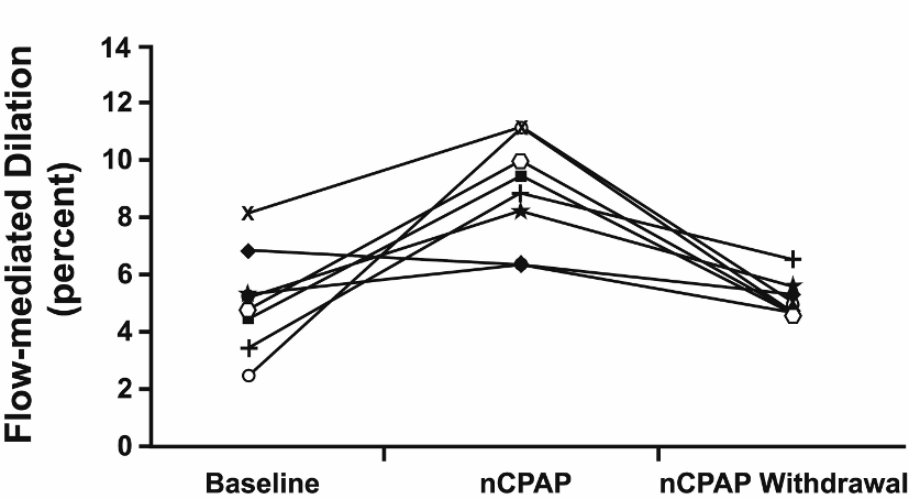

Figure 2. Flow–mediated dilation of the brachial artery in patients with obstructive sleep apnea before treatment (Baseline), after 4 weeks of treatment with nasal continuous positive airway pressure (nCPAP), and 1 week after treatment was temporarily discontinued (nCPAP Withdrawal). In most patients, flow-mediated dilation improved with treatment, an effect that was lost when treatment was withdrawn. From Ip et al (44); used with permission.

The functional consequences of OSA-induced endothelial dysfunction are incompletely understood; however, greatly attenuated hypoxic vasodilation in the forearm has been observed in these individuals (79) and in healthy human subjects exposed for 8 hours to hypocapnic hypoxia (31). In patients with OSA, attenuated hypoxic vasodilation was observed in the cerebral circulation, an effect that was reversed with CPAP treatment (26).

The mechanisms by which exposure to intermittent hypoxia produces endothelial dysfunction have been investigated in rat models. Tahawi et al. reported diminished acetylcholine-induced dilations in cremaster arterioles of rats exposed to episodic hypoxia (90). In this study, the constrictor response to acute NO synthase inhibition was smaller in hypoxic vs. control rats, suggesting that exposure to intermittent hypoxia lowers basal levels of NO. In our laboratory, we exposed rats to intermittent hypoxia (FIO_2=0.10 for 1 min; 15 times/hr; 12 hrs/day). After a 2-week exposure, the gracilis artery (a skeletal muscle resistance artery) and middle cerebral artery were isolated for performance of functional studies *in vitro*. In both vessels, acetylcholine-induced

vasodilations were greatly attenuated in arteries from hypoxia-exposed vs. normoxic control rats (Figure 3) (74). At the same time, dilator responses to sodium nitroprusside, an NO donor, were similar in the two groups, suggesting that chronic intermittent hypoxia impairs acetylcholine-induced vasodilation via reductions in the bioavailability of NO. In both the middle cerebral and gracilis arteries, the vasodilator responses to acute reductions in perfusate and superfusate PO_2 were virtually abolished.

Interestingly, blunted vasoconstrictor responsiveness to norepinephrine was also observed our rat model (73). Myogenic vasoconstriction in response to step increases in intralumenal pressure was also impaired; whereas Ang II-induced vasoconstriction was unaffected. In contrast, attenuated vasoconstrictor responses were not seen in rats that received tempol, a superoxide dismutase mimetic, during intermittent hypoxia exposure, suggesting that the observed impairment was caused by an excess of superoxide ion. In contrast to these findings, endothelin-induced vasoconstriction in the mesenteric circulation was enhanced, not blunted, in rats exposed to intermittent asphyxia (1). In the forearms of patients with OSA, Hedner and colleagues observed impaired norepinephrine-induced vasoconstriction (37) and enhanced Ang II-induced vasoconstriction (49).

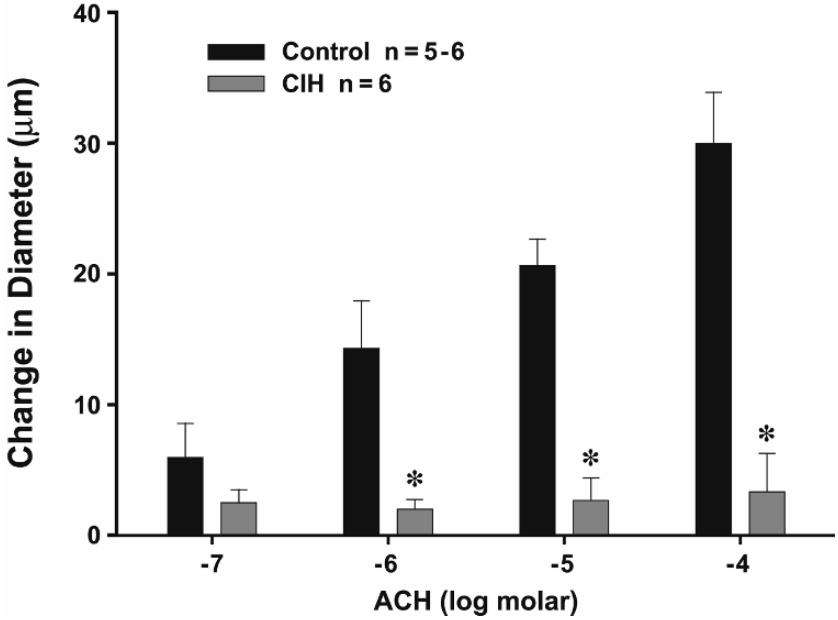

Figure 3. Acetylcholine (ACH)-induced vasodilations were greatly attenuated in gracilis arteries isolated from rats exposed to chronic intermittent hypoxia (CIH) vs. normoxia (Control) for 14 days. Data are presented as mean changes ±SE from the diameter measured before application of ACH. *P < 0.05, CIH vs. Control. From Phillips et al (74); used with permission.

Recent clinical observations point to oxidant stress as a trigger for intermittent hypoxia-induced vascular dysfunction. Circulating levels of NO derivatives were found to be depressed in patients with OSA (43). Increased production of superoxide by neutrophils (82) and increased levels of 8-isoprostane in breath condensate (8) were also observed. Intermittent hypoxia-induced generation of reactive oxygen species may alter vascular function by perturbing the balance between reactive oxygen species and NO in the endothelial cells (16). Angiotensin II, a potent mediator of oxidant stress (67), may contribute importantly to this process. A recent clinical study suggests that xanthine oxidase-derived superoxide plays an important role in causing intermittent hypoxia-induced endothelial dysfunction. In patients with OSA, flow-mediated dilation in the forearm was enhanced by allopurinol, a xanthine oxidase inhibitor (21).

Alterations in arterial wall structure and biomechanics.

Increased carotid intima-media thickness (59; 92) and increased arterial stiffness (72; 92) have been observed in individuals with OSA. In our rat model, vascular wall stiffness in gracilis arteries increased after a 14 day exposure to intermittent hypoxia (73). Precisely how intermittent hypoxia causes vascular remodeling is unknown; however, several mechanisms can be postulated.

OSA is associated with chronic inflammation, as evidenced by increased blood levels of C-reactive protein and various pro-inflammatory cytokines (83; 103). In animal models, continuous exposure to hypoxia triggers an inflammatory response in the microcirculation of several vascular beds (85). It is conceivable that the intermittent hypoxia of OSA has even greater pro-inflammatory effects, secondary to the generation of reactive oxygen species during hypoxia-reoxygenation cycles (93). Inflammatory cells contribute to vascular remodeling via secretion of enzymes that disrupt the balance between metalloproteinases and their inhibitors in extracellular matrix (45).

A number of mitogenic factors known to participate in vascular remodeling (e.g. vascular endothelial growth factor, basic fibroblast growth factor, platelet-derived growth factor) are upregulated during exposure to hypoxia (7; 15). In OSA patients, blood levels of NO and endothelin-I, endothelium-derived regulators of vascular stiffness with opposing actions, are decreased and increased, respectively (43; 71). Increased Ang II levels have been observed patients with OSA (60) and may play a role in intermittent hypoxia-induced vascular remodeling. In this regard, vascular Ang II is a well-established promoter of local vascular inflammation and remodeling (35; 105), and in the central nervous system, Ang II acts to increase sympathetic outflow (106).

Chronically elevated sympathetic nervous system activity (6) may contribute to vascular remodeling in patients with OSA. Increases in sympathetic outflow can evoke an inflammatory cascade in some organs and vascular beds (104) and catecholamines released as the result of sympathetic stimulation can induce vascular wall growth that is dependent on generation of reactive oxygen species (4). It has recently become evident that adventitial fibroblasts contribute to hypoxia-induced vascular remodeling (86). The release of ATP from adrenergic nerve terminals during hypoxia-induced sympathetic stimulation causes proliferation and migration of adventitial fibroblasts into the intimal and medial layers in pulmonary arteries (30), and may also be a stimulus for remodeling in the systemic circulation. In addition, the surges in sympathetic outflow

that accompany episodes of apnea produce cyclical increases in arterial pressure and blood flow. The cyclical stretch caused by these surges may trigger adaptations in endothelial cells, vascular smooth muscle, and extracellular matrix aimed at normalizing wall stress (94; 99).

ARE THE EFFECTS OF CONTINUOUS HYPOXIA DIFFERENT FROM THOSE OF INTERMITTENT HYPOXIA?

Humans experience continuous hypoxia during high altitude exposure and also in the advanced stages of pulmonary disease. In contrast to the many recent advances in our understanding of the effects of intermittent hypoxia, relatively little is known about the vascular consequences of continuous hypoxia. We do know that, in healthy humans, systemic arterial pressure rises during sojourns at high altitude (38; 100). This increase in arterial pressure was accompanied by activation of sympathetic vasoconstrictor outflow, an effect that persisted for several days after return to lower elevations (38). The mechanisms underlying this sustained sympathetic activation are unclear; however, they probably involve adaptations other than enhanced chemoreflex sensitivity, because the high nerve traffic observed after return to sea level was not affected by supplemental oxygen (38). Consistent with this finding, previous investigators determined that only intermittent (and not continuous) hypoxia caused sensory long-term facilitation of carotid sinus nerve activity (68).

Marked sympathetic activation has also been observed in patients with chronic respiratory failure (40). Interestingly, patients who are chronically hypoxemic secondary to respiratory failure do not seem to be at increased risk for developing systemic hypertension. A potential explanation for these incongruent findings is that exposure to continuous hypoxia greatly attenuates vascular responses to increases in sympathetic outflow (41) and to other constrictor stimuli (see below).

In rats, exposure to continuous hypoxia causes attenuated reactivity to vasoconstrictor stimuli in several vascular beds (9; 17; 66). Following 4 weeks of continuous hypoxia, Walker and colleagues observed attenuated increases in total peripheral vascular resistance in response to graded infusions of phenylephrine, Ang II, and arginine vasopressin (14). These investigators subsequently observed, after only 48 hours of continuous hypoxic exposure, that both agonist-induced and myogenic constrictions in isolated mesenteric arteries were impaired (17; 19). This hypoxia-induced depression of vasoconstrictor reactivity, which was caused by persistent hyperpolarization of vascular smooth muscle, is dependent on an intact endothelium (17; 19). Nitric oxide, carbon monoxide, and EET derived from the endothelium all appear to contribute to this impairment (18; 20; 34; 63).

SUMMARY

Over time, exposure to intermittent and sustained hypoxia causes vascular dysfunction. Angiotensin II and reactive oxygen species acting at the carotid body, the central nervous system, and the resistance arterioles contribute importantly to the adverse vascular sequelae of long-term hypoxic exposure. Hypoxia-induced vascular dysfunction may impair blood flow regulation and compromise tissue perfusion and oxygen delivery during acute episodes of OSA. Moreover, these impairments may have important functional consequences during exercise, when an intricate interplay between neural, mechanical, and local chemical control mechanisms in the peripheral vasculature is required to maintain blood supply to active muscle. Given the negative influence that intermittent and continuous hypoxia exert on many of these responses, the possibility exists that the decrements in physical performance at high altitude and those observed in patients with chronic pulmonary disease are caused, at least in part, by hypoxia-induced impairments in blood flow regulation.

Once the mechanisms of hypoxia-induced alterations in vascular structure and function are understood, strategies can be developed to reverse or prevent them. Such research has relevance to hypertension and other important cardiovascular sequelae of OSA, chronic respiratory failure, and high altitude exposure.

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Chapter 7

ANGIOTENSIN-INDUCED HYPOXIA IN THE KIDNEY: FUNCTIONAL AND STRUCTURAL CHANGES OF THE RENAL CIRCULATION

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Abstract: Recent studies emphasize the role of chronic hypoxia in the kidney as a final common pathway to end-stage renal disease (ESRD). Hypoxia of tubular cells leads to apoptosis or epithelial-mesenchymal transdifferentiation. This in turn exacerbates fibrosis of the kidney with loss of peritubular capillaries and subsequent chronic hypoxia, setting in train a vicious cycle whose end point is ESRD. To support this notion, our studies utilizing various techniques such as hypoxia-sensing transgenic rats revealed hypoxia of the kidney in various disease models.

While fibrotic kidneys with advanced renal disease are devoid of peritubular capillary blood supply and oxygenation to the corresponding region, imbalances in vasoactive substances and associated intrarenal vasoconstriction can cause chronic hypoxia even at the early phase of kidney disease. Among various vasoactive substances, local activation of RAS is especially important because it can lead to constriction of efferent arterioles, hypoperfusion of postglomerular peritubular capillaries, and subsequent hypoxia of the tubulointerstitium in the downstream compartment. Recent studies using BOLD-MRI showed an immediate decrease of oxygen tension in the kidney after angiotensin II infusion. In addition, angiotensin II induces oxidative stress via activation of NADPH oxidase. Oxidative stress damages endothelial cells directly, causing the loss of peritubular capillaries. Oxidative stress also results in relative hypoxia due to inefficient cellular respiration. Thus, angiotensin II induces renal hypoxia via both hemodynamic and nonhemodynamic mechanisms.

While the beneficial effects of blockade of RAS in kidney disease are, at least in part, mediated by amelioration of hypoxia, recent studies have also elucidated the mechanism of hypoxia-induced gene regulation via the HIF-HRE system. This has given hope for the development of novel therapeutic approaches against hypoxia in the kidney.

Key Words: chronic kidney disease, kidney failure, angiotensin II, oxidative stress, hypoxia inducible factor

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INTRODUCTION

An undeniable epidemic is the marked increase in the number of patients with end stage renal disease (ESRD) (14) and chronic kidney disease (CKD) that has become a major health issue in various parts of the world. In addition, the economic burden of ESRD treatment to patients, their families, the public, and the nation is also enormous. The seriousness of these problems of CKD required the worldwide nephrology community to rededicate itself to the retardation and prevention of the progression of all forms of renal disease.

There are many different kidney diseases, including glomerulonephritis, diabetic nephropathy, and hypertensive nephrosclerosis. However, once renal damage reaches a certain threshold, progression of renal disease is consistent, irreversible, and largely independent of the initial insult.

Close pathological analysis has revealed better correlation of functional impairment of the kidney with the degree of tubulointerstitial damage than with that of glomerular injury. Now it is widely recognized that the final common pathways which mediate the deterioration of kidney failure is to be found in the tubulointerstitium.

Although the kidneys receive a very high blood flow, oxygen extraction in the kidney is low. Furthermore, reabsorption of a large fraction of the sodium and water filtered by the glomeruli is necessary to maintain homeostasis in the body. This reabsorption process is driven by active transport and uses a large amount of oxygen. Renal tissue oxygen tension in the kidney ranges from 40–50 mmHg in the outer cortex to 10–15 mmHg in the inner medulla, and the relatively high oxygen levels in the cortex, which contains the major sites of sodium reabsorption, namely the proximal tubule and thick ascending limb of the loop of Henle, supports the high oxygen requirement in this section. As a consequence, the kidneys are particularly susceptible to hypoxic injury. As a final common pathway to ESRD, recent studies emphasize chronic hypoxia in the tubulointerstitium (8, 28, 31, 32, 34, 36).

THE MICROVASCULATURE OF THE KIDNEY

In the kidney, most afferent arterioles arise from the interlobular arteries. Except for branches that go toward the pelvic mucosa, all blood from the interlobular and arcuate arteries is directed into the glomerular capillary bed. Glomerular capillaries merge together again at the vascular pole to form the efferent arterioles. Glomerular efferent arterioles from cortical glomeruli supply a fine capillary plexus which lies around the tubules beneath the capsule and in the areas of cortex between the interlobular vessels (Fig 1).

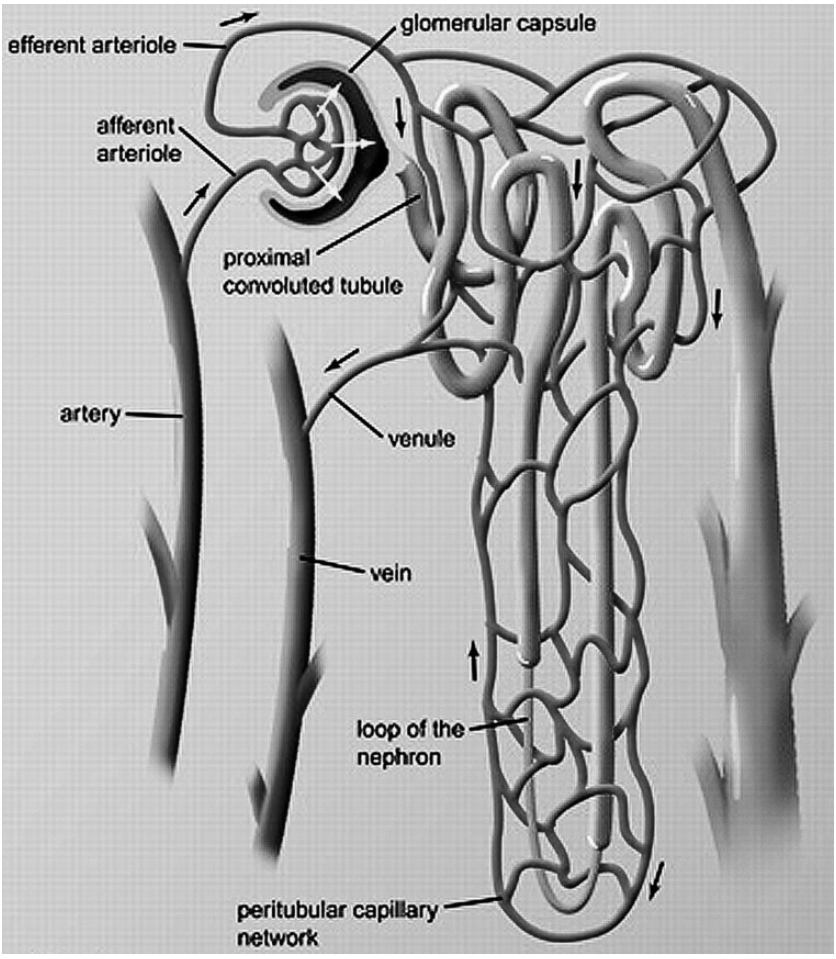

Figure 1. The microvasculature of the nephron. The peritubular capillary plexus is fed by glomerular efferent arterioles and supplies oxygen to tubular and interstitial cells. While structural changes of this plexus by angiotensin II leads to loss of capillaries and fibrosis, constriction of efferent arterioles by angiotensin II also results in hypoxia via reduction of peritubular capillary blood flow.

The efferent vessels of the juxtamedullary glomeruli supply the subcortical capillary plexus in addition to dividing into vasa recta which enter the medulla. The peritubular capillary plexus surrounds tubules and offers oxygen to tubular cells and interstitial cells. Thus, whenever blood flow in the peritubular capillary is impaired, the kidney suffers from hypoxia.

Peritubular capillary loss leading to hypoxia of the corresponding regions has been demonstrated in a variety of disease models. Ohashi from Yamanaka's group emphasized a pathogenic role of loss of peritubular capillaries in a rat anti-glomerular basement membrane-induced glomerulonephritis model and experimental obstruc-

tive nephropathy (37, 38). Kang from Johnson's group described inverse correlation between peritubular capillary density and interstitial fibrosis in the aging kidney and in the remnant kidney model (12, 13). Kairaitis from Harris' group showed peritubular capillary loss preceding increased cortical hypoxia in a model of advanced adriamycin nephrosis (11). Yuan from Woolf's group detected cortical tubular atrophy, interstitial fibrosis, and loss of peritubular capillaries in association with tissue hypoxia in a model of folic acid nephropathy (59). Sun and colleagues demonstrated the reduction of peritubular capillary density with the subsequent tubulointerstitial fibrosis in a model of aristolochic acid nephropathy (46).

Namikoshi from Kashihara's group recently analyzed the number of peritubular capillaries in patients with variable severity of IgA nephropathy (30). The numbers of peritubular capillaries were significantly lower in patients with more severe tubulointerstitial injury.

CHRONIC HYPOXIA OF THE KIDNEY: ANIMAL MODELS

Matsumoto from our group employed intravital microscope to measure capillary blood flow in the kidney of experimental rats (25). This system is composed of a pencil lens probe and CCD-assisted videomicroscopic system, allowing us to evaluate renal microcirculation. She showed stagnation of peritubular capillary blood flow at the early phase of a model of progressive glomerulonephritis induced by uni-nephrectomy and repeated injection of anti-Thy1 antibodies. The decrease in peritubular capillary blood flow was observed well before the development of structural capillary injury. In order to detect hypoxia in the kidney of this model, we employed pimonidazole, a reagent which binds to hypoxic cells. Pimonidazole uptake is detected by immunohistochemical methods. Stagnation of peritubular blood flow was associated with hypoxia in the kidney.

Diabetic kidneys are also hypoxic. We investigated a strain of SHR/NDmc-cp as a model of type II diabetic nephropathy. In this strain, the genetic mutation of the leptin receptor generates obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. Pimonidazole staining again revealed that the kidney of SHR/NDmc-cp is hypoxic (10). Hypoxia of the diabetic kidney was also demonstrated by BOLD-MRI technique (41). This method utilizes field distortion by deoxyhemoglobin in the magnetic field, which appears as BOLD contrast in the resulting images. The kidney of streptozotocin (STZ)-induced diabetic rats was hypoxic from an early stage.

HYPOXIA-SENSING TRANSGENIC ANIMALS

Cells are endowed with a system against hypoxia, which is composed of hypoxia-inducible factor, HIF, and hypoxia-responsive element, HRE. In normoxia, HIF1-α is hydroxylated, which enables the von Hipped-Lindau protein to bind to HIF-1α, culminating in degradation. Under hypoxic conditions, however, HIF-1α escapes degra-

dation, binds to the constitutively expressed HIF-1β, and exerts its hypoxic response through binding to the cis- consensus HIF-binding site, HRE. A variety of genes such as EPO and VEGF are regulated by HIF. We utilized this system to establish hypoxia-sensing transgenic rats.

With these rats, we challenged the hypothesis that tubulointerstitial hypoxia occurs in the kidney during renal disease and modifies the pathogenic progression. In the puromycin nephrosis model, hypoxic tubules were visualized diffusely in the cortex at both 1 and 2 weeks (49). In the remnant kidney model, on the other hand, hypoxic areas started to extend from the outer medulla to the cortex at week 1, becoming more pronounced at week 4. These rats also enabled us to reveal chronic hypoxia of the aging kidney (54). We identified the age-related expansion of hypoxia in all areas of the kidney. Expansion was most prominent in the cortex. The degree of hypoxia was positively correlated with the age-related tubulointerstitial injury.

Safran from Kaelin's group recently published hypoxia-sensing transgenic mice with a similar strategy (42). They created a mouse that ubiquitously expresses a bioluminescent reporter consisting of firefly luciferase fused to a region of HIF that is sufficient for oxygen-dependent degradation. IVIS imaging camera showed bioluminescence of these mice after administration of luciferin. Of note, the kidney is already hypoxic under normoxic conditions. When these mice were placed in a low oxygen environment, a 5- to 10-fold increase in light emission was observed, and the kidney became severely hypoxic.

ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM IN CHRONIC KIDNEY DISEASE

Activation of the renin-angiotensin system is often observed in patients with CKD and leads to increased synthesis of angiontesin II. Angiotensin II constricts precapillary arterioles and increases blood pressure. Aldosterone release from the adrenal cortex is stimulated by angiotensin II and causes renal sodium retention and expansion of circulating blood volume. In addition, the angiotensin II contents in renal tissues are much higher than can be explained on the basis of equilibration with the circulating concentrations, and it is now apparent that activation of the local renin-angiotensin system plays a crucial role in kidney disease (16).

STRUCTURAL CHANGES OF THE RENAL CIRCULATION BY ANGIOTENSIN II

Angiotensin II plays a crucial role in chronic hypoxia of the kidney. Angiotensin II damages the vasculature and induces fibrosis. Angiotensin II stimulates expression of a major fibrogenic cytokine, TGF-β, in the kidney and upregulates receptors for TGF-β *(58)*. Angiotensin II can directly phosphorylate Smads without inducing TGF-β. Recent data also suggested that other components of the renin-angiotensin system including

angiotensin III, renin, and aldosterone also activate the TGF-β system. In addition, angiotensin II induces epithelial-mesenchymal transdifferentiation and induces renal fibrosis (21). Fibrotic kidneys are devoid of peritubular capillary blood, and the corresponding region becomes hypoxic.

We previously demonstrated loss of peritubular capillaries in angiotensin II–infused rats by staining with an endothelium-specific antibody, JG-12 (44). Capillary loss was ameliorated by administration of angiotensin receptor blocker (ARB), olmesartan. Recent studies utilizing another endothelium-specific antibody, RECA-1, confirmed loss of peritubular capillaries in angiotensin II–infused rats (15). Loss of peritubular capillaries was again mitigated by ARB. Loss of peritubular capillaries and progression of fibrosis by angiotensin II was confirmed by quantitative histological analysis.

Even in the presence of peritubular capillaries, interstitial fibrosis impairs tubular oxygen supply because the extended distance between capillaries and tubular cells reduces efficiency of oxygen diffusion. A fibrogenic response by angiotensin II in turn would lead to the obliteration of peritubular capillaries and decrease blood supply. Subsequent hypoxia induces transdifferentiation of tubular cells into myofibroblasts (epithelial mesenchymal transdifferentiation), predisposing the kidney to fibrosis (23). Hypoxia also induces apoptosis of renal tubular and endothelial cells via the mitochondrial pathways (47, 48, 50), rendering a vicious cycle of fibrosis and regional hypoxia.

FUNCTIONAL CHANGES OF THE RENAL CIRCULATION BY ANGIOTENSIN II

At an early stage of renal disease before development of the structural changes, angiotensin II induces hypoxia via functional changes.

Angiotensin II constricts efferent arterioles and decreases blood flow in post-glomerular peritubular capillaries. Recent studies by Nishiyama's group visualized the superficial peritubular capillaries directly using an intravital fluorescence videomicroscope system (18). They evaluated the peritubular capillary blood flow by analyzing the velocity of fluorescein-labeled erythrocytes. Intravenous administration of angiotensin II (50 ng/kg/min, 10 min) decreased the peritubular capillary erythrocyte velocity by 37% in rats.

In the study by Schachinger and colleagues, 6 healthy male volunteers were examined by BOLD-MRI after intravenous administration of angiotensin II, norepinephrine, and sodium nitroprusside (43). Angiotensin II caused a shortening of T2* between 6% and 10%, indicating sodium nitroprusside and norepinephrine did not alter the renal BOLD signal. The renal BOLD response to angiotensin II appeared with short onset latency (as early as 10 seconds after angiotensin II bolus administration), suggesting that this response is a consequence of altered perfusion of the peritubular capillary plexus.

In addition, angiotensin II induces hypoxia of the kidney via another functional change. Angiontesin II induces oxidative stress via activation of NADPH oxidase. Oxidative stress alters oxygen metabolism and oxygen availability.

Reactive oxygen species react with nitric oxide (NO) to form peroxynitrite and thus decrease the bioavailability of NO. In mitochondria, NO at low concentrations binds to and inhibits cytochrome c oxidase and essentially blocks the binding of oxygen through the formation of nitrosyls, thus interfering with normal mitochondrial respiration. Therefore, a decrease in NO induces dysregulation of mitochondrial respiration, leading to inefficient oxygen usage. The potency of NO-mediated inhibition of renal oxygen usage was demonstrated by the pioneering study of Laycock et al., in which they showed that administration of a nitric oxide synthase (NOS) inhibitor increased the overall renal oxygen usage (20). An isoform of NOS responsible for the oxygen consumption remains controversial. Deng from Blantz's group showed that neuronal (n) NOS inhibition increases the oxygen costs of kidney function (6). In contrast, studies by Adler and colleagues studied renal cortex from mice deficient (-/-) in endothelial (e) NOS and found that NO production by eNOS is responsible for regulation of renal oxygen consumption in mouse kidney (1).

Palm and colleagues measured oxygen tension in the diabetic kidney using Clark-type microelectrodes. Renal oxygen tension was lower throughout the renal parenchyma of STZ-induced diabetic rats when compared to control rats (40). Early transplantation of pancreatic islets to STZ-treated animals prevented a decrease in renal oxygen tension. Physiological studies utilizing kidney slice cultures showed an increase of renal oxygen consumption in diabetic rats (39). Treatment with α-tocopherol throughout the course of diabetes prevented diabetes-induced disturbances in oxidative stress, oxygen tension and consumption. Welch from Wilcox's group also showed that reduced efficiency of renal oxygen usage for tubular sodium transport in the clipped kidney of the early 2-kidney, 1-clip angiotensin II-dependent model was restored by the superoxide dismutase mimetic tempol *(*55). They recently extended these findings utilizing rats which received prolonged angiotensin II administration. Angiotensin II reduced efficiency of renal oxygen usage for tubular sodium transport, resulting in a decrease in the oxygen tension in the proximal tubule and throughout the cortex. Tempol blunted or prevented all these effects of angiotensin II (56). Recent studies by Adler and Huang also showed that NO bioavailability is impaired in spontaneously hypertensive rats (SHR) owing to an angiotensin II-mediated increase in superoxide production in association with enhanced expression of NAD(P)H oxidase components (3). Their studies utilizing tempol showed that decreased NO availability in the SHR kidney resulted in increased oxygen consumption (2). Studies utilizing tissue from Fischer 344 rats at different ages revealed that impaired production of NO in aging leads to decreased regulation of oxygen consumption in the kidney (4). These results suggest that oxidative stress relates to augmented oxygen consumption in the kidney.

HIF-ACTIVATING THERAPY AGAINST RENAL HYPOXIA

Chronic hypoxia in the kidney is a final common pathway to ESRD, and therapeutic approaches targeting hypoxia in the kidney should be effective in patients with CKD. A promising approach to protecting tissues against hypoxia might be activation of a "master gene" switch, HIF.

Our recent studies revealed a postnatal biological role of HIF-2α in the kidney (17*)*. We induced the well-established ischemia reperfusion injury model of the kidney in HIF-2α knockdown (kd) mice. These mice show normal development, while expression of HIF-2α mRNA in the kidney was reduced to 50% without any changes of HIF-1α mRNA expression. While ischemia impaired renal function in both wild type and HIF-2α kd mice, HIF-2α kd mice had a significantly higher BUN and more severe histological changes. Evaluation of oxidative stress markers showed greater oxidative stress in HIF-2α kd mice, and we examined expression of anti-oxidative enzyme genes in these mice. Expression of SOD1, SOD2 and GPX1 genes was significantly lower in HIF-2α kd kidney. On the other hand, expression of HO-1, a target gene of HIF-1α, was not different between wild type and HIF-2α kd kidneys. We speculated that HIF-2α in the kidney endothelium is responsible for regulation of oxidative stress. HIF-2α can be found not only in endothelial cells but also in interstitial cells including inflammatory cells in the kidney. Our bone marrow transplantation experiments showed that the knockdown of HIF-2α in inflammatory cells was not involved in susceptibility to renal ischemia reperfusion injury. Knockdown of HIF-2α gene was achieved by inserted neomycin gene sandwiched between two loxP sequences. We restored the expression of HIF-2α specifically in endothelium by intercrossing HIF-2α kd mice with Tie1-Cre mice. Susceptibility of HIF-2α knockdown mice to renal ischemia was restored in HIF-2α kd::Tie1-Cre mice. This result clearly demonstrated a specific role of HIF-2α in endothelium, raising a possibility that stimulation of HIF can be a powerful tool to protect the vasculature under hypoxic conditions.

To support this notion, previous studies by our group and others demonstrated that stimulation of HIF with cobalt chloride is effective in a variety of kidney disease models. The summary of these studies is in Table 1. In addition, treatment of STZ-induced diabetic rats with vitamin C and cobalt chloride resulted in partial restoration of anti-oxidative stress enzyme activities in the kidney (59). Cobalt chloride also increased the secretion of angiogenin and VEGF by cultured proximal tubular epithelial cells (29). While others utilized cobalt protoporphyrin and reported successful treatment of kidney disease models such as the 2K1C model of renovascular hypertension (5), rapamycin-induced renal dysfunction in ischemia-reperfusion injury (9), and STZ-induced diabetic nephropathy (7), it should be noted that the beneficial effect of cobalt protoporphyrin is likely to be mediated by HIF-HRE independent up-regulation of HO-1 (22).

Table 1. HIF-activation by cobalt in kidney disease models.

Model		
Progressive anti-Thy glomerulonephritis	Tanaka et al. 2005 (51)	Improvement of tubulointerstitial injury Decrease in the number of apoptotic cells
Remnant kidney	Tanaka et al. 2005 (52)	Improvement of tubulointerstitial injury Decrease in the number of apoptotic cells preservation of peritubular capillary networks
Cisplatin nephropathy	Tanaka et al. (53)	Improvement of tubulointerstitial injury Decrease in the number of apoptotic cells
Habu snake venom-induced glomerulonephritis with co-administration of angiotensin II	Kudo et al. (19)	Improvement of renal function
Ischemia-reperfusion injury	Matsumoto et al. (26)	Improvement of renal function Improvement of tubulointerstitial injury

ANGIOTENSIN BLOCKADE AS A THERAPEUTIC MODALITY AGAINST RENAL HYPOXIA

Although HIF stimulation is a promising future therapy, today's best modality to treat kidney disease is blockade of the renin-angiotensin system (33). One important mechanism of blood pressure-independent renoprotection by blockade of the renin-angiotensin system is preservation of peritubular capillary perfusion.

Norman and Fine studied anesthetized adult rats (35). Cortical microvascular oxygenation was measured on the surface of the exposed kidney utilizing the porphyrin phosphorescence technique. There was a slow decline in cortical oxygenation in control animals over the 3-hour experimental period. Administration of ACEi or ARB at the beginning of the experimental period completely abrogated this decline.

We demonstrated a decrease in blood flow in peritubular capillaries and subsequent hypoxia in a very early phase of remnant kidneys (week 1) (24). These changes were associated with narrowing and distortion of peritubular capillaries, but not with a de-

crease in the number of peritubular capillaries. Physiologic perfusion status of the peritubular capillary network was evaluated by lectin perfusion and Hoechst dye diffusion techniques. Treatment of these animals with ARB restored blood flow in peritubular capillaries and improved oxygenation of the kidney.

Subsequently, Zhang and colleagues studied peritubular capillary loss and tubulointerstitial hypoxia in remnant kidney rats at the later time points of week 3, week 6 and week 12 (61). Peritubular capillary loss and tubulointerstitial hypoxia were persistent in the process of developing interstitial fibrosis at all these time points.

Long-term administration of ARB in type 2 diabetic rats also resulted in restoration of oxygenation in the kidney (10). We and others demonstrated anti-oxidative stress effects of ARB. Because angiotensin II induces oxidative stress via activation of NADPH oxidase, blockade of the receptor inhibits oxidative stress. Furthermore, chemical structures of ARB inhibit in vitro oxidative stress by chelating transition metals and inhibiting various oxidative steps in a receptor-independent manner (27, 45). Thus, mechanisms of the improved oxygenation by blockade of the renin-angiotensin system include both hemodynamic changes via dilatation of glomerular efferent arterioles and efficient oxygen usage via amelioration of oxidative stress. To support this notion, Welch from Wilcox's group demonstrated that administration of ARB, candesartan, improved inefficient utilization of oxygen for sodium transport in the SHR kidney (56).

CONCLUSION

Chronic hypoxia in kidney disease serves as a final common pathway leading to ESRD. Angiotensin II induces hypoxia of the kidney via structural microvasculature damage and fibrotic changes (Figure 2). Angiotensin II leads to constriction of glomerular efferent arterioles, resulting in reduction of peritubular capillary blood flow and subsequent hypoxia in the corresponding region. Furthermore, angiotensin II induces oxidative stress, which in turn consumes NO and results in inefficient oxygen usage. Therapeutic approaches utilizing ACEi or ARB against this final common pathway is effective in a broad range of renal diseases.

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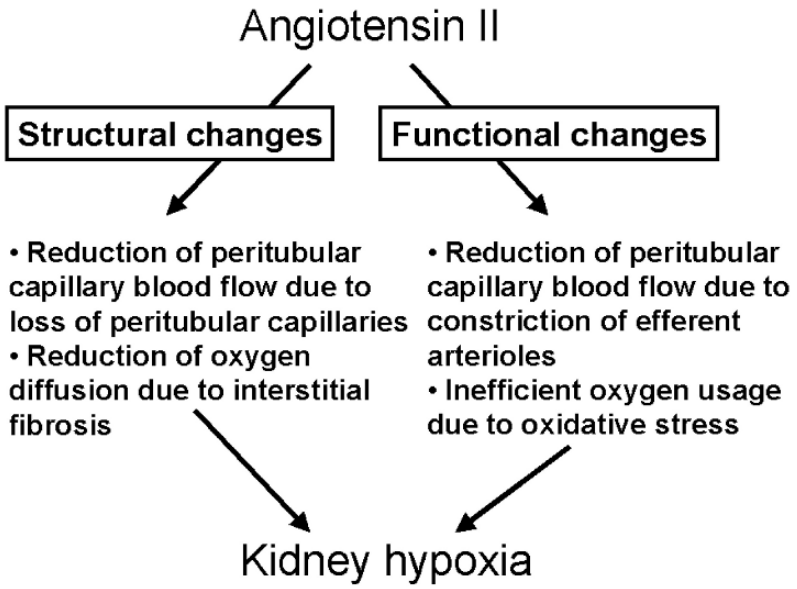

Figure 2. Mechanisms of renal hypoxia induced by angiotensin II. Angiotensin II induces hypoxia of the kidney via structural microvasculature damage and induction of fibrosis. Angiotensin II also leads to constriction of glomerular efferent arterioles and reduces peritubular capillary blood flow. Furthermore, angiotensin II induces oxidative stress, which in turn results in inefficient oxygen usage.

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Chapter 8

ROLE OF REACTIVE OXYGEN SPECIES IN CHRONIC HYPOXIA-INDUCED PULMONARY HYPERTENSION AND VASCULAR REMODELING

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Abstract: Pulmonary hypertension is a life-threatening disease process that affects adults and children. Pediatric patients with lung diseases that can be complicated by alveolar hypoxia, such as bronchopulmonary dysplasia (BPD), are at risk for developing pulmonary hypertension, which leads to right heart failure and greatly increases morbidity and mortality. We review the evidence that reactive oxygen species (ROS) are generated by pulmonary vascular wall cells in response to a hypoxic exposure, and that this response contributes to chronic hypoxic pulmonary hypertension. We summarize the accumulating data implicating NADPH oxidase as a major source of O_2^- responsible for vascular remodeling and hypertension. We also consider the effects of chronic hypoxia on the clearance of O_2^- by superoxide dismutases, specifically extracellular superoxide dismutase, which is highly expressed in the pulmonary artery. We review the role of the activated vascular adventitial fibroblast in the generation of ROS and in the pathogenesis of vascular remodeling, and provide a rationale to consider the role of the activated fibroblast and ROS in hypoxic pulmonary hypertension using a clinically relevant bovine model of neonatal chronic hypoxic pulmonary hypertension.

Key Words: superoxide, NADPH oxidase, pulmonary vascular remodeling, adventitia, adventitial fibroblast

INTRODUCTION

Pulmonary hypertension is a life-threatening disease process that affects adults and children. Pediatric patients with hypoxic lung diseases such as bronchopulmonary dysplasia (BPD), cystic fibrosis or diffuse interstitial fibrosis are at risk for developing pulmonary hypertension, a complicating condition which can lead to right heart failure and greatly increases morbidity and mortality. (48) The pathologic changes in the pulmonary circulation of the infants and children with pulmonary hypertension are characterized by striking structural remodeling in the pulmonary arteries (PA),

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particularly in the media and adventitia (Fig 1). The causes of abnormal pulmonary vascular development and structural remodeling in the pediatric population are poorly understood. Chronic hypoxic exposure induces profound pulmonary vascular remodeling in most immature animal models, and is thus a highly useful model to study the mechanisms driving pulmonary vascular remodeling and pulmonary hypertension.(Fig 2)(48) Thus, investigations in these models aimed at better understanding the mechanisms responsible for chronic hypoxia-induced pulmonary vascular remodeling are important and necessary to design new therapeutic strategies to treat this life-threatening complication.

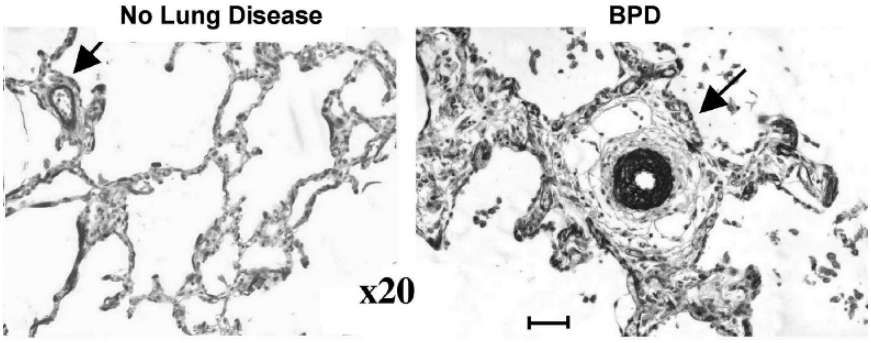

Figure 1. Lung pathology in an infant who succumbed to BPD with associated pulmonary hypertension. There is extensive adventitial and medial remodeling in the pulmonary artery of an infant dying from BPD and pulmonary hypertension (right). The left shows normal pulmonary artery architecture in an infant dying of a non-pulmonary process. Lungs are immunostained for α-smooth muscle actin (brown stain).

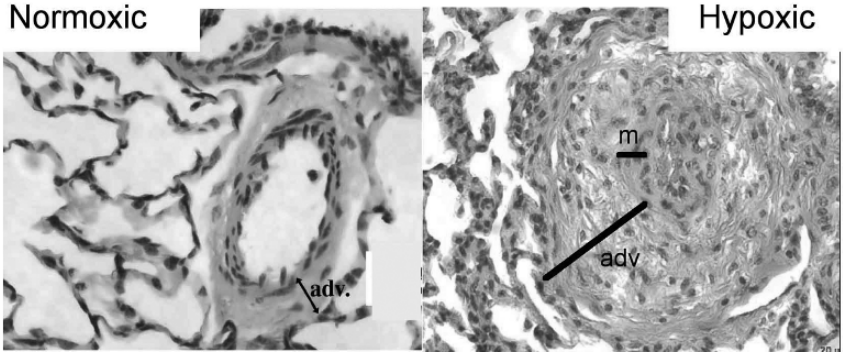

Figure 2. Distal PA structural remodeling in a chronically hypoxic calf with pulmonary hypertension: significant adventitial component. Neonatal calves were exposed since day of life 1 in a hypobaric hypoxia chamber to a simulated altitude of 15,000 ft (430 mm Hg). This barometric pressure is sufficient to generate severe pulmonary hypertension in the neonatal calf by 14 days. The right panel shows a hematoxylin & eosin stain of lung sections from a 2 week chronically hypoxic calf. A lung section of an age-matched normoxic calf is shown for comparison on the left. Each section contains a small distal pulmonary artery. There is extensive adventitial remodeling in the distal PA of the chronically hypoxic calf.

PRODUCTION OF REACTIVE OXYGEN SPECIES IN CHRONIC HYPOXIA

It is increasingly apparent that reactive oxygen species (ROS), including superoxide (O_2^-), contribute to chronic hypoxic pulmonary hypertension. The issues surrounding ROS and hypoxia are complex and controversial, particularly the role of ROS in acute hypoxic pulmonary vasconstriction.(13,23,32,55, 56) Chronic hypoxia causes both vasoconstriction and PA vascular remodeling, and ROS impacts both processes. Over the past 5 years, research studies from our laboratory and others have shown that ROS production is increased in the PA under hypoxic conditions. (8,14,15,16,26,34,57) In one recent study, Fresquet et al demonstrated that rats exposed to chronic hypobaric hypoxia for 2 weeks showed evidence of increased total O_2^-, as detected by dihyroethidine staining in frozen lung sections.(8) We previously showed that the production of extracellular O_2^- the pulmonary artery of rats increased in rats exposed to 3 days of hypoxia compared with normoxic rats, detected by superoxide dismutase-inhibitable cytochrome c reduction.(34) Similar increases in O_2^- by cytochrome c reduction have been reported in the pulmonary artery of chronically hypoxic mice.(26) Markers of oxidative stress are also increased in the lung in response to chronic hypoxia. Sprague-Dawley rats exposed to 3 weeks of hypobaric hypoxia showed increased lung lipid peroxidation.(14) Consistent with this finding, we have new evidence in calves that chronic hypoxia also increased protein oxidation, measured by a two-fold increase in protein carbonyl levels in lung homogenates. The detection of ROS along with more stable markers of oxidation collectively provide solid evidence for increased ROS production in the lung vasculature in response to a low oxygen environment.

SOURCES OF CHRONIC HYPOXIA-INDUCED ROS IN THE PULMONARY ARTERY

The source(s) and cell type(s) responsible for ROS production in the hypoxic pulmonary artery are not well defined.(50) Sources of O_2^- include mitochondrial electron transport chain, xanthine oxidase, cytochrome P-450, NOS, and NADPH oxidase. It is well established that mitochondria are a major cellular source of intracellular O_2^-, though their contribution to ROS production under hypoxic conditions is highly debated.(55,56) One study indicates that mitochondrial O_2^- contributes to sustained (120 minutes) hypoxic pulmonary vasoconstriction in isolated perfused rabbit lungs.(58) Another study implicated xanthine oxidase, as xanthine oxidase enzymatic activity increased in lungs of chronically hypoxia rats, while allopurinol blunted pulmonary hypertension and pulmonary vascular remodeling.(14) O_2^- is produced from endothelial nitric oxide synthase (eNOS) when the 2 enzymatic domains of eNOS are uncoupled, and we have found that endothelial NOS is an important source of extracellular O_2^- in the PA of the normoxic adult rat but not the 3 day hypoxic rat.(34) Uncoupling of eNOS is developmentally regulated in the fetal and 4 week old lamb, contributing to age-dependent differences in O_2^- production in the PA.(29) Vascular NADPH oxidases

are also now recognized as an important source of O_2^- and are receiving increasing attention with regard to their contribution to changes in function and structure of the vessel wall in diverse disease processes.

NADPH oxidase has been increasingly implicated in vascular remodeling and hypertension in both the systemic and pulmonary circulation.(1,5,12) A number of studies demonstrate that NADPH oxidase is an important source of O_2^- in the PA under hypoxic conditions. Several of these studies further implicate gp91phox-containing NADPH oxidase (Nox2) as the major NADPH oxidase isoform responsible for the vascular production of O_2^-.(1,8,26,57,59) To test the hypothesis that NADPH oxidase, specifically Nox2, -derived ROS contribute to hypoxic pulmonary hypertension, Liu et al found that mice lacking the membrane-associated subunit of NADPH oxidase, gp91phox (Nox2) were protected from pulmonary vascular remodeling and pulmonary hypertension.(26) The Nox2 homolog, Nox4, has also been identified as an abundant NADPH oxidase in human PA smooth muscle cells and shown to be important in smooth muscle cell proliferation.(50) O_2^- derived from NADPH oxidase may uncouple eNOS domains, further increasing O_2^- production in the PA. The role of NADPH oxidase in the pulmonary circulation of the immature animal has not been well-defined. However, in fetal lambs, an NADPH oxidase produces extracellular O_2^- within the adventitia and media and contributes to ductal ligation-induced pulmonary hypertension by inactivating NO.(4,21) In addition, ROS generated from NADPH oxidase mediate PA smooth muscle cell proliferation in cell culture models relevant for neonatal pulmonary hypertension.(29,41) Overall, these data indicate that there are multiple potential sources of ROS, with substantial data implicating an important role for NADPH oxidase in hypoxia-induced pulmonary hypertension.

REACTIVE OXYGEN SPECIES GENERATED WITHIN THE ADVENTITIA CONTRIBUTE TO CHRONIC HYPOXIC PULMONARY HYPERTENSION

ROS generated under hypoxic conditions may contribute to pulmonary hypertension by promoting both vascular remodeling and vasoconstriction. Effects of ROS may be via direct reactions of O_2^-, or H_2O_2 derived from O_2^-, which function as paracrine signaling molecules.(25) O_2^- can generate lipid oxidation products, e.g. minimally modified low-density lipoproteins, that function as intracellular signaling molecules (3). Alternatively, as noted above, effects may be mediated by reactions between O_2^- and NO, altering NO-mediated vasodilation or other NO signaling events (37,44).

While much of the work of vascular biologists has focused on the role of endothelial cells and smooth muscle cells in disease pathogenesis, increasingly, new data indicate that the adventitia, the outer layer of the blood vessel, contributes significantly to vascular remodeling and vascular function.(48, 46). Multiple cell types populating the adventitia may contribute to the production of adventitial ROS. These cell types include the resident adventitial fibroblast, inflammatory cells recruited to the vessel wall, adventitial nerves, and vaso vasorum endothelial cells, as well as adjacent epi-

thelial cells. In the neonatal calf, the PA adventitial fibroblast has been identified as a key cell type that responds early and dramatically to chronic hypoxia *in vivo* to mediate changes within the pulmonary adventitia. (2,6,46) The adventitial fibroblast in response to environmental stresses such as hypoxia or overdistension produces ROS via adventitial NADPH oxidase, leading to "activation" of the cell.(17,39,53) The activated fibroblast then undergoes a phenotypic switch characterized by proliferation, matrix protein production, differentiation into a myofibroblast, and acquisition of migratory characteristics that collectively can contribute to thickening and fibrosis of the adventitia and even media.(30,44,45, 48) New data from our laboratory demonstrate that circulating monocytic progenitor cells are recruited to the PA adventitia and contribute to adventitial remodeling.(9,47) These cells are another potential source of hypoxia-induced adventitial ROS, supported by a recent publication showing bone-marrow derived progenitor cells express NADPH oxidase.(9,43) Nitroblue tetrazolium (NBT), which stains blue when reduced by O_2^-, has been used to detect O_2^- and localize its production to the adventitia of blood vessels.(34,40) We can reproduce these findings in freshly isolated pulmonary artery segments from chronically hypoxic calves, localizing the reduction of NBT predominantly to the adventitia. Thus, there is a strong rationale to further interrogate how overproduction of ROS within the adventitia in response to chronic hypoxia can promote pulmonary hypertension through increases in vasoconstriction and vascular remodeling.

EXTRACELLULAR SUPEROXIDE DISMUTASE IS HIGHLY EXPRESSED WITHIN THE ADVENTITIA

The production of ROS in the adventitia is balanced by endogenous antioxidant defenses, which include the superoxide dismutases. The superoxide dismutases are an important family of antioxidant enzymes that catalyze the rapid dismutation of O_2^- to H_2O_2 (~$6.7 \times 10^{-9} M^{-1}s^{-1}$).(28,35,36,49) Three isoforms, cytosolic Cu,Zn-SOD, mitochondrial Mn-SOD and extracellular EC-SOD, have been identified and characterized.(18) EC-SOD is abundant in the lung and vasculature and is the only extracellular enzymatic defense against O_2^-. The biologic impact of EC-SOD has been directly attributed to its specific localization to the outer adventitial and medial layers of the blood vessel, where it can limit reactions of extracellular O_2^- and preserve NO bioactivity in blood vessels.(18) We have previously shown that EC-SOD expression and activity are tightly regulated in the developing lung and changes in O_2 environment in the perinatal period can disrupt the normal secretion of active EC-SOD in the lung.(33) Though it has been implicated in systemic hypertension as well as fibrotic and inflammatory lung diseases, the contribution of EC-SOD to chronic hypoxic pulmonary hypertension has not been tested.

REACTIVE OXYGEN SPECIES AS SIGNALLING MOLECULES; REGULATION OF REDOX-SENSITIVE TRANSCRIPTION FACTORS IN CHRONIC HYPOXIC PULMONARY HYPERTENSION

Reactive oxygen species do not simply produce oxidative damage to cellular structures, but are now well recognized to function as signaling molecules to sense and respond to cellular stress. Effects of ROS may be via direct reactions of O_2^-, or H_2O_2 derived from O_2^-, which function as paracrine signaling molecules.(25) O_2^- can generate lipid oxidation products, e.g. minimally modified low-density lipoproteins, that function as intracellular signaling molecules(3). Alternatively, effects may be mediated by reactions between O_2^- and NO, limiting NO-mediated vasodilation or other NO signaling events.(37,44) Reactive oxygen species modulate many signaling pathways, including the expression of redox-sensitive transcription factors.(24) One classic example of a redox-sensitive hypoxia-inducible transcription factor and gene target is HIF-1α and erythropoietin in the kidney. Other redox-regulated transcription factors include AP-1, NF-κB, and Egr-1. Egr-1 is a member of the early growth response gene family of zinc finger transcription factors.(31,51). It is now recognized as a master regulator of multiple genes relevant in the pathogenesis of cardiovascular diseases.(10,60). These genes include genes related to growth, inflammation, matrix proteins, and coagulation. Egr-1 and Egr-1-responsive genes have been implicated in numerous cardiovascular and pulmonary diseases including atherosclerosis, ischemia-reperfusion, COPD, lung fibrosis, lung inflammation and hypoxia-induced pulmonary vascular remodeling and hypertension.(42,60,61) In chronically hypoxic calves and bovine PA adventitial fibroblasts, our group has shown that Egr-1 increases in the lung, localizing to adventitia; and hypoxia-exposed fibroblasts isolated from the adventitia increase Egr-1 mRNA, protein and binding activity.(2,11) Furthermore, Egr-1 increases growth related genes, cyclin D and EGFR, and inhibition of Egr-1 attenuates hypoxia-induced fibroblast growth phenotype. A number of studies indicate that SOD activity can modulate the upregulation of transcription factors. Mn-SOD and EC-SOD activity have both been reported to influence the hypoxic-induction of HIF-1α. For example, in stably transfected cells, increased levels of Mn-SOD activity decreased hypoxia-induced HIF-1α expression (54). In mice lacking EC-SOD, Suliman et al found that EC-SOD expression was necessary for hypoxic induction of renal HIF-1α and erythropoietin. (52,62) A substantial amount of work is still required to understand how an imbalance in oxidants and antioxidants can alter transcription factors, including Egr-1, and their downstream genes important in pulmonary vascular remodeling and pulmonary hypertension.

ANTIOXIDANT STRATEGIES ATTENUATE CHRONIC HYPOXIC PULMONARY HYPERTENSION

The role for ROS in the pathogenesis of hypoxic pulmonary hypertension is further substantiated by a number of reports of different antioxidant strategies that protect

against hypoxia-induced pulmonary hypertension. Early studies showed protection against hypoxic pulmonary hypertension in rats treated with dimethylthiourea.(20,22) N-acetylcysteine protects against hypoxic pulmonary hypertension in rats, and one study in chronically hypoxic rats showed protection with the SOD mimetic, tempol (7,19). Interestingly, several studies suggest that ROS are produced early in response to hypoxia, as treatment with N-acetylcysteine in the first week, or allopurinol in the first three days of hypoxia protects against late hypoxic pulmonary hypertension.(14,19) There is limited data examining the endogenous antioxidant defenses in the lung following chronic hypoxia. We have new evidence in the weanling rat model of chronic hypoxic pulmonary hypertension that protein levels of the important vascular antioxidant enzymes, extracellular superoxide dismutase (EC-SOD), decreased in the lung at 1 and 2 weeks of hypoxia. (Fig 3) This finding is consistent with the observation that lung EC-SOD can be inactivated by exposure to hyperoxia, another injury model associated with oxidative stress. (27,38) A recent manuscript further showed that the lamb model of ductal ligation-induced pulmonary hypertension is attenuated in lambs treated with intratracheal recombinant SOD, providing strong evidence for ROS in neonatal pulmonary hypertension.(21) Additional research is necessary to test whether an imbalance in production and clearance of extracellular superoxide contributes to the structural remodeling in chronic hypoxic pulmonary hypertension in humans. (Fig 4) These studies will provide the basis for future human clinical trials in a range of scenarios associated with hypoxic lung diseases to improve health outcome for patients with these difficult and serious problems.

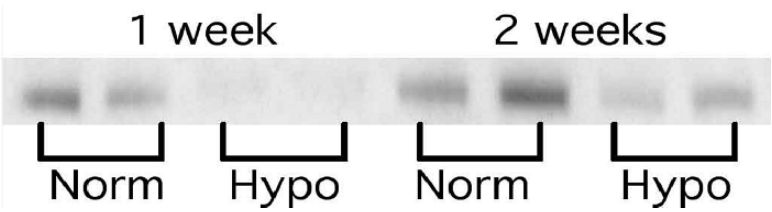

Figure 3. Lung EC-SOD protein expression decreased in chronically hypoxic rats. Weanling Wistar Kyoto rats exposed to hypobaric hypoxia at 17,000 ft (395 mmHg) develop pulmonary hypertension and pulmonary vascular remodeling, similar to the chronically hypoxic neonatal calf. EC-SOD protein expression was measured in the lungs of Wistar-Kyoto rats exposed to 1 and 2 weeks of hypoxia by Western blot analysis using a monoclonal mouse anti-rat EC-SOD peptide antibody (kindly provided by Dr. Louise Giles, University of Manitoba) EC-SOD protein expression decreased in lungs of immature exposed to 1 or 2 weeks of hypoxia.

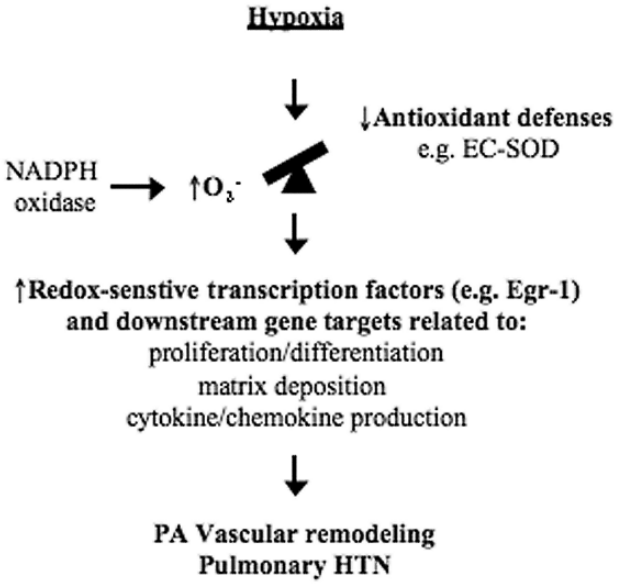

Figure 4. Working hypothesis. We hypothesize that hypoxia disrupts the balance between the production of O2- by NADPH oxidase and its clearance by EC-SOD within the pulmonary artery adventitia. We further hypothesized that excess O2- generated in the hypoxic lung activates cells within the adventitial layer of the pulmonary artery to upregulate redox-sensitive transcription factors, such as Egr-1, which, in turn, stimulates downstream genes important in causing neonatal chronic hypoxia-induced pulmonary vascular remodeling and pulmonary hypertension.

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Chapter 9

HYPOXIA AND PLACENTAL REMODELLING

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Abstract: In the first trimester of pregnancy fetal trophoblast cells invade the maternal uterine spiral arteries leading to loss of the vascular cells from the vessel wall and remodelling of the extracellular matrix. This is crucial to ensure that sufficient blood can reach the developing fetus. Impaired arterial remodelling is a feature of the major pregnancy pathologies pre-eclampsia and fetal growth restriction. Despite its importance, little is known about the regulation of this process. We have shown, using in vitro culture models and ex vivo explant models, that trophoblast cells play an active role in remodelling spiral arteries, and have implicated apoptotic events in this process. Further we have shown that trophoblast-derived factors such as Fas-ligand, tumor necrosis factor-related apoptosis inducing ligand (TRAIL) are important regulators of this process. The oxygen tension within the uteroplacental environment will vary with gestational age and will depend on the extent of trophoblast invasion and artery remodelling. Fluctuations in oxygen tension may be an important determinant of cellular events both during invasion towards uterine vessels and during the remodelling process. The components of this process known to be regulated by oxygen are reviewed, including lessons that can be learned from pregnancies at high altitude. In addition, data on the effect of varying oxygen tension on trophoblast production of pro-apoptotic factors and susceptibility of vascular smooth muscle cells to induction of apoptosis are described.

Key Words: trophoblast, spiral artery, apoptosis, pregnancy

INTRODUCTION

Adaptation of the uterine blood vessels is of fundamental importance to ensure a healthy and successful pregnancy. In the first trimester of pregnancy the process of placentation is instigated following implantation of the blastocyst into the uterine wall. Fetal cytotrophoblast stem cells either fuse to form a multinucleated syncytiotrophoblast or differentiate into extravillous trophoblast. Syncytiotrophoblast line the surface

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of floating villi which are bathed in maternal blood where they function as a barrier between the maternal and fetal cells and mediate gas and nutrient exchange to the developing fetus. Extravillous trophoblasts invade from anchoring villi into the uterine wall (interstitial invasion) and its blood vessels (endovascular invasion) as far as the myometrial segments. The walls of the uterine spiral arteries are transformed by extravillous trophoblasts which temporarily replace the endothelial lining, cause a loss of musculoelastic tissue in the vessel walls and deposition of fibrinoid material (17, 51). These crucial alterations to the maternal vessels, occurring as a normal part of pregnancy, result in the creation of a high-flow, low-resistance circulation that increases maternal blood flow to the placental villi at the maternal-fetal interface (reviewed in (52)). Little is known as to how these processes are regulated in normal pregnancies.

The pathway taken by the extravillous trophoblast to reach the lumen of the spiral arteries remains a topic debated in the field (40, 52) with the relative contributions of cells that have invaded interstitially (intravasation) versus endovascularly (extravasation) warranting further clarification. In addition, some of the changes to the vessels will occur independently of the presence of trophoblast cells; for example endothelial vacuolation and swelling of the vascular smooth muscle layer precede the trophoblast-associated remodelling events and have recently been termed decidua-associated remodelling (52).

The pivotal importance of these changes to the uterine vessels in the establishment and maintenance of a successful pregnancy is illustrated when they fail to occur or occur to a significantly reduced extent. Defective remodelling of the spiral arteries is associated with pregnancies complicated by pre-eclampsia and fetal growth restriction (6). Pre-eclampsia is a disorder which affects 5-7% of pregnancies worldwide, is responsible for considerable perinatal mortality and morbidity and carries health implications in adult life, including increased risk of hypertension, heart disease and diabetes (3). In pre-eclamptic women only the decidual segments of the spiral arteries show normal remodelling (5).

The temporal and spatial control of cellular interactions in the complex uteroplacental environment remains to be fully resolved. In order to reach the uterine vessels, trophoblasts must be motile, invasive and resist cell death, in an environment rich in pro-apoptotic factors. If the cells successfully reach the spiral arteries, then interactions with the vascular cells and remodelling of the vessel must be tightly regulated. It remains to be determined what may account for the differences in remodelling seen between normal and complicated pregnancies; however, a number of studies have highlighted the influence that the local oxygen tension has in the control of these events.

OXYGEN TENSION IN THE PLACENTA

A subtle balance has to exist between the needs of the fetus and placenta in regard to oxygen supply and the potential danger of oxidative stress and the production of oxygen free radicals (35). As extravillous trophoblasts invade the spiral arteries plugs of trophoblast cells form in the vessels (8, 30). As a consequence maternal blood will

be prevented from entering the intervillous space. Evaluation of placental oxygen *in vivo* showed that placental pO_2 before 11 weeks gestation was <20 mmHg (equivalent to 2-3% O_2), 2.5 times lower than the decidual pO_2 (36, 37). As a consequence, the fetoplacental unit will develop in a relatively hypoxic environment in the first trimester. In the absence of maternal blood flow to the intervillous space, endometrial secretions will provide nutrition for the fetoplacental unit (27). Blood flow to the intervillous space will occur after 10-12 weeks when the trophoblast plugs loosen and at this stage oxygen levels surrounding the placental villi will reach ~55 mmHg (~8% O_2) (37).

Trophoblast cells must differentiate from a proliferative to an invasive phenotype in order to access the uterine vessels. There is evidence that low oxygen tension plays a role in regulating these events, although studies have given conflicting results. It has been suggested that in low oxygen concentrations trophoblasts are promoted to proliferate while in high oxygen concentrations cells will differentiate into an invasive phenotype. Explant cultures of first trimester chorionic villi under different oxygen tensions showed that those cultured in 2-3% oxygen were more proliferative and had increased numbers of extravillous trophoblasts growing out from the villous tips than those cultured in 20% oxygen (12, 21). However, in direct contrast, others have suggested that there is a decrease in proliferation and extravillous trophoblast outgrowths in explants cultured at 1.5% oxygen compared to 8% oxygen (32). Some of these differences may be attributed to interpretation of whether trophoblast outgrowth in these models represents increased proliferation or invasiveness (discussed in (33)). An interesting paradox is discussed by Pijnenborg et al (52) who question how trophoblast reach the spiral arteries in order to plug them if they are proliferative rather than invasive at this stage and raise the possibility that implantation is associated with a temporary state of high oxygen, allowing the initial trophoblast invasion.

Other studies have suggested that low oxygen is a stimulus for extravillous trophoblasts to adopt an invasive phenotype; for example, when cytotrophoblast cell lines were cultured under 1% oxygen, invasion through Matrigel was stimulated compared to normal culture conditions (23, 24) although using the same cell line Kilburn et al showed increased proliferation and reduced invasion at 2% oxygen (44).

Following remodelling of the spiral arteries and dissolution of the trophoblastic plugs there will be a steep rise in the oxygen tension. It has been proposed that this will lead to an overall state of oxidative stress or fluctuations in oxygen concentrations analogous to hypoxia-reperfusion within the placental environment (28). Indeed, there is a peak in the expression of markers of oxidative stress in trophoblasts co-incident with the onset of the circulation (34). Premature entry of maternal blood into the intervillous space secondary to shallow trophoblast invasion and insufficient plugging of the vessels has been implicated in early pregnancy loss (34).

TROPHOBLAST SENSING OF OXYGEN TENSION

The functional effect of changes in oxygen tension will be dependent on the ability of the trophoblast to sense these changes. Considerable progress has been made in elucidating how this sensing takes place and is regulated, with many studies focussing

on the hypoxia inducible factor (HIF) system. In low oxygen tension the labile α subunit of HIF stabilizes and is translocated to the nucleus where it forms a heterodimer with the constitutively expressed β subunit. This heterodimer will then bind to hypoxia response elements (HREs) in the promoter region of a number of genes, thereby inducing transcription. Under normoxic conditions HIF-α is targeted for degradation by the ubiquitin-proteasome pathway following binding of the von Hippel-Landau tumor suppressor protein (pVHL). In the placenta HIF-1α is strongly expressed from 5 weeks of gestation but has virtually disappeared by 12 weeks (11). Evidence that the HIF system is important is illustrated by the fact that mice lacking expression of HIF1-β or pVHL have faulty placentation and offspring die in utero (1, 22). These factors have also been implicated in the regulation of trophoblast differentiation and invasion in vitro (20, 31).

HYPOXIA AND PRE-ECLAMPSIA

Many studies have provided evidence that placental hypoxia contributes to the pathology of pre-eclampsia (reviewed in (35)). Since remodelling of the spiral arteries is defective in this condition, perfusion of the intervillous space will be impaired compared to normal pregnancies leading to the assumption that a continued hypoxic placenta may be an important contributory factor in the pathology of the disorder. It may however be the case that intermittent perfusion of the intervillous space leads to fluctuating oxygen tensions and an ischaemia-reperfusion type of injury (28). This disturbance in the oxidant-antioxidant balance will leave the tissue more vulnerable to the deleterious effects of oxygen free radicals. A number of consequences have been suggested, including increased apoptosis in placental villi and increased shedding of apoptotic or necrotic trophoblast, which could traffic to and affect the maternal circulation and contribute to systemic inflammation in the mother (29, 56).

REGULATION OF SPIRAL ARTERY REMODELLING

A balance between factors that both promote and inhibit vascular cell survival is important in the maintenance of vessel integrity. A number of mechanisms could play a role in the loss of the vascular cells from uterine spiral arteries, including migration, dedifferentiation, and programmed cell death (apoptosis). These are not mutually exclusive events, and it is likely that two or more precisely orchestrated processes have a part to play. It is also clear that, whatever the mechanism, spiral artery remodelling takes place over a period of weeks and is tightly regulated to prevent sudden loss of vessel integrity. We hypothesized that active apoptotic processes may be induced in endothelial and vascular smooth muscle cells while they still line the vessel. Apoptosis is an asynchronous process and affected cells are rapidly removed through phagocytosis by either neighboring cells (19) or infiltrating macrophages; hence, even in tissues undergoing rapid remodelling, where the rate of cellular turnover is high, the detection

of apoptotic cells experimentally is low (4).

Studies of spiral arteries have been confined primarily to immunohistochemical analysis of placental bed biopsies (60) while *in vitro* studies have been hampered by the lack of suitable models to directly examine cellular interactions during invasion. To address these problems and to investigate the mechanisms responsible for these essential vascular changes we developed *in vitro* models of spiral artery invasion and remodelling (13, 14).

Our studies have shown that extravillous trophoblasts can induce apoptosis in both endothelial cells and vascular smooth muscle cells, in both co-culture and in an explant model of spiral artery remodelling (2, 25). These results were confirmed in studies by Red-Horse et al using a novel technique where placental villi were implanted into the mammary fat pads of *Scid* mice and invasive trophoblasts were shown to specifically induce apoptosis in arterial smooth muscle and endothelial cells (54). Induction of apoptosis by trophoblasts is not without precedent since trophoblast-induced apoptosis has been observed in epithelial cells, which were subsequently removed by phagocytosis (39). Furthermore, trophoblasts can induce apoptosis in lymphocytes thereby conferring a degree of immune privilege on the placenta (38).

A number of mechanisms trigger apoptosis in vascular cells including loss of adhesion due to changes in integrin expression and stimulation of the tumor necrosis factor (TNF) receptor family including Fas (CD95), TNF-R1 or TNF-related apoptosis-inducing ligand (TRAIL) receptors. Although the initiation of these pathways may differ they converge towards the activation of common effector molecules, including the caspases, proteolytic enzymes that catalyze cleavage of key cellular proteins. Binding of Fas ligand (FasL) to cell-surface Fas in many cells leads to apoptosis of the Fas-bearing cell (57). FasL on trophoblasts can induce apoptosis in lymphocytes (41) and we have shown that trophoblast induction of vascular cell apoptosis is partly mediated by Fas/FasL interactions (2, 25). This effect appears to be through both the release of soluble factors from the extravillous trophoblast and via cell-cell contact. In addition, we have recently implicated TRAIL/TRAIL receptor interactions (42) in the effect on vascular smooth muscle cells, and soluble HLA-G, a soluble form of the HLA class Ib molecule expressed by extravillous trophoblast, as a pro-apoptotic factor for endothelial cells (18).

Using our *in vitro* model of spiral artery remodelling we have investigated both the interstitial and endovascular invasion routes. Trophoblast invasion of vessels by both routes was higher at 17% than 3% oxygen (16). It may be that there are two different stages of regulating invasion: firstly the effect of oxygen on trophoblast proliferation/invasion of the decidua and subsequently on trophoblast invasion and interaction with the vessel. This latter interaction could also be influenced by the responses of the vascular cells to changes in oxygen tension. Entry into the vessel (by intravasation or extravasation), interactions with the endothelial cells as they temporarily co-exist in the vessel wall, induction of apoptotic changes in endothelial or vascular smooth muscle cells by production of soluble pro-apoptotic factors or by direct cell-cell contact could all be considered as potential targets for regulation by oxygen tension. An additional level of complexity is added by the fact that the local oxygen tension will be highly variable depending on the location of the trophoblast/vascular cell interaction in rela-

tion to any trophoblastic plugs. Gradients in oxygen tension may regulate the movement of the endovascular trophoblast, which occurs in a retrograde manner within the spiral artery lumen.

To address whether changes in oxygen tension can affect sensitivity of vascular cells to trophoblast derived pro-apoptotic factors the effect of the hypoxia mimetic agent cobalt chloride on TRAIL-induced human vascular smooth muscle cell apoptosis was assessed (Figure 1A). We have previously shown that TRAIL induces vascular smooth muscle cell apoptosis, that extravillous trophoblasts produce TRAIL and that spiral artery vascular cells express TRAIL receptors (42). In the current studies, mimicking hypoxic conditions had no effect on either basal or TRAIL-induced apoptosis. To extend these studies vascular smooth muscle cells were cultured under different oxygen tensions and apoptosis was induced by TRAIL or activation of the Fas receptor. Apoptosis was assessed by western blot analysis for caspase-dependent PARP cleavage (Figure 1B). There was no difference in the level of apoptosis induced in response to TRAIL or Fas activation if the cells were cultured at 20% oxygen, at 3% hypoxia or under conditions modelling hypoxia-reoxygenation. Sensitivity of vascular smooth muscle cells in our *in vitro* cultures does not appear to be influenced by changing oxygen tension. These cells are derived from human aortic vascular smooth muscle cells; it remains to be determined whether the same response is seen with spiral artery vascular cells. In our *ex vivo* spiral artery remodelling studies, vessels are dissected from biopsies obtained at elective caesarean section and will be subjected to oxidative stresses during sample collection, as will primary first trimester trophoblasts (7). Although extrapolation of *in vitro* data to the *in vivo* situation must be done with caution, in the placental field these are often the most informative experiments available since functional *in vivo* experiments are impossible to perform.

To determine whether trophoblast production of pro-apoptotic factors such as TRAIL is regulated by oxygen, extravillous trophoblasts were cultured under different oxygen tensions. At 20% oxygen cell-associated TRAIL production was induced in trophoblast by interferon-γ (IFNγ) and TNFα (Figure 2). However, when cells were incubated at 3% oxygen this response was completely blocked. Cells which had undergone hypoxia/reoxygenation had partially recovered the ability to produce TRAIL. This may be important because, as the trophoblast are invading towards the uterine vessels, they will be under a relatively hypoxic environment and production of pro-apoptotic factors such as TRAIL could be detrimental to surrounding cells. On reaching the artery and being subjected to oxygen concentrations that may then be analogous to hypoxia/reoxygenation, the trophoblast is now in the correct location to switch on production of a pro-apoptotic factor, which plays an important role in the selective, local remodelling of the vessel.

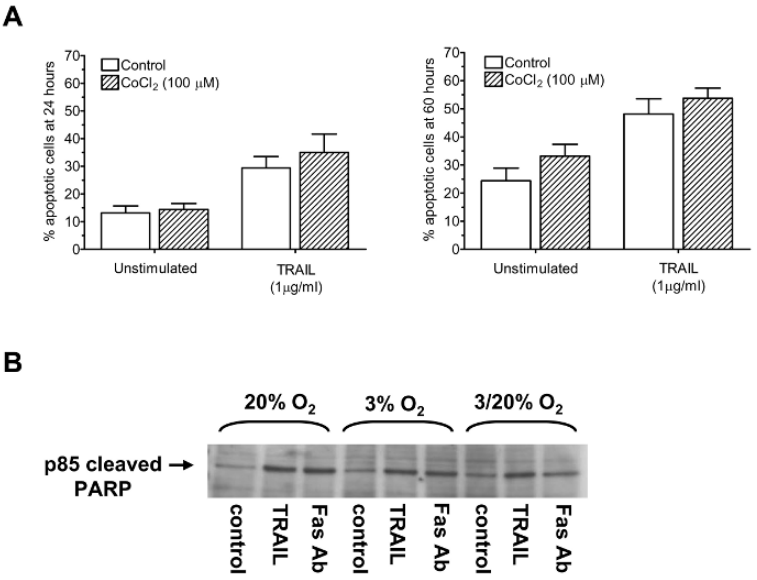

Figure 1. Effect of hypoxia on vascular smooth muscle cell apoptosis. **A.** Effect of cobalt-chloride induced hypoxia on human vascular smooth muscle cell apoptosis induced by TRAIL. HASMC were cultured as previously described (25) and plated at 3.6 x 10^4 cells/well in a 12-well plate. After 24h, the medium was replaced to include TRAIL (1μg/ml) and/or cobalt chloride (100μM). Apoptosis was monitored by time-lapse microscopy using an Olympus IX70 inverted microscope with a motorized stage and cooled charge-coupled device camera enclosed in a humidified chamber at 37°C with 5% CO_2 as previously described (2). Images were taken at 15 minute intervals and time-lapse sequences were analysed using Image Pro Plus (Media Cybernetics, MD). Forty cells were scored in each field of view, and the time at which apoptotic morphology was first observed was recorded (characterised by membrane blebbing, cytoplasmic shrinkage, nuclear condensation, a phase bright appearance and the formation of membrane blisters). TRAIL significantly induced HASMC apoptosis after 24h ($p<0.03$, n=4, Mann Whitney *U* test) and 60h stimulation ($p<0.03$, n=4, Mann Whitney *U* test). The presence of cobalt chloride had no significant effect on the extent of apoptosis. Results shown are mean + sem of 4 independent experiments. **B.** Effect of hypoxia or hypoxia/reoxygenation on human vascular smooth muscle cell apoptosis induced by TRAIL or Fas activation. HASMC were cultured in the presence or absence of TRAIL (0.2μg/ml) or a Fas-activating antibody (0.25μg/ml) as previously described (25). Cells were either maintained under normal culture conditions at 37 °C in humidified atmospheric air with 5% CO_2 (20% oxygen; pO_2 ~140 mmHg) or in 3%O_2/89% N_2/5% CO_2 (hypoxia; pO_2 ~20 mmHg) for 28h or at 3% oxygen for 24h followed by 20% oxygen for 4h (hypoxia-reoxygenation). After treatments HASMC were lysed as previously described (25). Equal amounts of protein (25 μg) were separated by SDS-PAGE on 10% gels and then transferred to PVDF membranes overnight. Membranes were blocked in Tris-buffered saline (TBS) containing 1% (w/v) BSA, and then probed with rabbit anti-human cleaved PARP (Promega, 1:1000) prepared in TBS + 0.05% (v/v) Tween 20 with 0.1% (w/v) BSA added, followed by HRP-conjugated secondary antibody. Following washing, proteins were detected by enhanced chemiluminescence. Results shown are representative of 3 separate experiments.

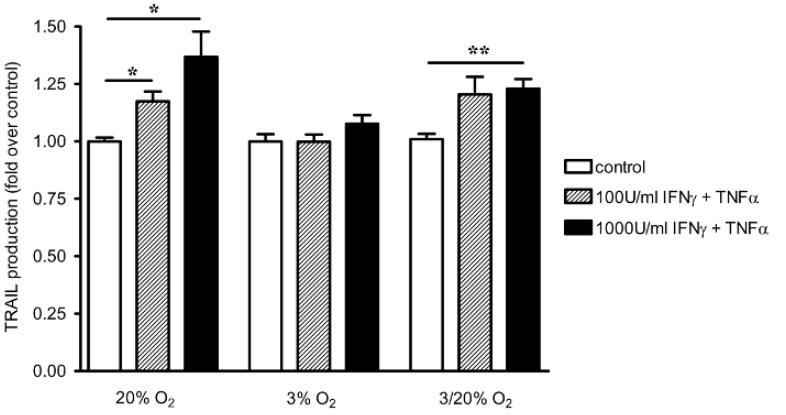

Figure 2. Effect of hypoxia or hypoxia/reoxygenation on extravillous trophoblast production of the pro-apoptotic cytokine TRAIL. SGHPL-4 cells (a trophoblast cell line derived from primary first trimester extravillous trophoblasts) were cultured as previously described (2) in the presence or absence of IFNγ (100U/ml or 1000U/ml) and TNFα (30ng/ml). Cells were either maintained under normal culture conditions at 37 °C in humidified atmospheric air with 5% CO_2 (20% oxygen; pO_2 ~140 mmHg) or in 3%O_2/89% N_2/5% CO_2 (hypoxia; pO_2 ~20 mmHg) for 28h or at 3% oxygen for 24h followed by 20% oxygen for 4h (hypoxia-reoxygenation). After treatments cells were lysed, standardised for protein content by Bradford assay and analysed for cell-associated TRAIL by ELISA according to the manufacturer's instructions (BD Biosciences). Results represent mean + sem from three individual experiments. *$p<0.04$, **$p<0.01$, Mann Whitney *U* test.

It is interesting to note that remodelling of veins in the uterine wall, which establish venous return, is limited to their termini, suggesting that extravillous trophoblast will target spiral arteries in preference to veins. It is highly likely that the differences in oxygen tension will play a role in this targeting, in addition to factors such as chemokines and ephrins (53).

LESSONS FROM HIGH ALTITUDE PREGNANCIES

Trophoblast remodelling of spiral arteries at high altitude is restricted (58), the incidence of pre-eclampsia elevated (43, 50) and birth weight is lower (26, 45). The literature strongly supports hypoxia as the likely stimulus for altitude pregnancy pathologies.

Although placental oxygen tensions have not been directly measured in high altitude pregnancies, there is evidence to suggest that placental oxygen delivery is lower than at sea level. In healthy pregnancies at sea level oxygen delivery to the uterus is protected by increased ventilation, blood volume, cardiac output and uteroplacental blood flow and lower systemic blood pressure. These cardiovascular adaptations differ significantly at high altitude; hypoxic ventilatory response and systemic blood pressure

are greater, and blood volume, cardiac output, and uteroplacental artery blood flow are less. Therefore, oxygen delivery to the placenta is reduced in high altitude pregnancies. Reduced maternal oxygen content is directly related to lower birth weight at high altitude (reviewed in (48)).

Less oxygen delivery at altitude is likely to result in the percent oxygen during 10 and 12 weeks gestation (approximately 3 and 8%, respectively at sea level) to be reduced to values such as 1 and 7% (12, 21). Because placental development is closely linked to oxygen availability, the reduction in oxygen delivery to the placenta may be a key factor in altering placental development at high altitude. Although the difference in percent oxygen reaching the placenta may be small (sea level vs. altitude), results from *in vitro* studies indicate that trophoblast phenotype differs between incubation in 1% as compared to 2% oxygen (23, 24, 44). Impairing trophoblast phenotypic change (from proliferative to invasive) likely reduces the formation of trophoblastic plugs and the associated surge in placental oxygenation that has been hypothesized to contribute to placental vascular development (34).

Trophoblast respond to hypoxia by activating the HIF-1 pathway; with a large amount of HIF-1 activity early in the first trimester and very little HIF-1 activity by the early second trimester (11). We reported that there is less HIF-1-DNA binding in placentas from healthy pregnancies at high as compared to low altitude (59). Although an unexpected finding, we hypothesized that placentas from healthy pregnancies at altitude have adapted to hypoxia, thus when hypoxia occurs during vaginal delivery there is less HIF-1 activation than at low altitude. HIF-1 transcriptionally mediates expression of proteins integrally involved in uteroplacental vascular remodelling, essentially governing trophoblast phenotype during hypoxia (1, 15).

We reported a reduction in uteroplacental vascular remodelling in healthy pregnancies at altitude (58). Uteroplacental vessels at altitude were composed of endothelium and smooth muscle cells in the presence of interstitial, but not endovascular trophoblasts. These data suggest a reduction in uteroplacental vascular flow, however there was an associated increase in the number of uteroplacental vessel entering the basal plate. The greater number of vessels may be an adaptation by healthy placentas at altitude to protect the placental oxygen supply. Indeed, in subsequent experiments total placental glutathione was elevated while succinate was reduced at altitude (58). Considering that glutathione is more dependent on oxygen content, while elevated succinate is a reflection of pO_2 (10, 49), the greater number of uteroplacental arteries may be improving oxygen content, but pO_2 would remain unchanged.

Uteroplacental artery remodelling is a key factor in determining the delivery of blood to the placenta and fetal capillary and villous development is a key factor in delivering oxygen from the placenta to the fetus. Fetoplacental vascularity refers to the capillary system within the villi that transports material to and from the fetus. Maternal blood pools in the intervillous space surrounding the villi, where exchange of oxygen, nutrients and waste products occurs with the fetoplacental capillaries within the villi. Villous vascularization is reported to improve at high vs. low altitude, although data regarding the specific morphology is variable; with increased capillary diameter, increased capillary length densities and increased capillary volume reported (9, 46, 55, 58). In pre-eclamptic pregnancies at sea level villous capillary development has

been reported to be impaired by reduced surface area, reduced capillary length density and/or greater trophoblast thickness (9, 47). Essentially, fetoplacental vascularization is improved in healthy pregnancies at high altitude and impaired in pre-eclamptic pregnancies at low altitude.

Uteroplacental vascularity and fetoplacental vascularity are greater at altitude, although uteroplacental remodelling is diminished in high altitude pregnancies. Metobolomic data from high and low altitude placentas indicating elevated membrane biosynthesis (greater phosphomonoester to phosphodiester ratio) (59), supports the morphometric data, suggesting greater placental vascularity in high as compared to low altitude placentas.

Placentas from high altitude healthy pregnancies have some morphologic characteristics of pre-eclamptic placentas (reduced uteroplacental artery remodelling) and some characteristics suggesting adaptation to the hypoxia of high altitude (greater uteroplacental artery number and fetoplacental vascular development). Discovering more about the pathogenesis of pre-eclampsia at altitude, where a 3-fold higher incidence is seen, may help to further understand the disease at any altitude.

Further investigation of placental development, in particular revealing which crucial uterine vessel remodelling events are faulty early on in pregnancies destined to become pre-eclamptic, is clearly warranted.

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Chapter 10

EPITHELIAL SODIUM CHANNELS IN THE ADULT LUNG – IMPORTANT MODULATORS OF PULMONARY HEALTH AND DISEASE

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Abstract: Absorption of excess fluid from the airways and alveolar lumen requires active vectorial transepithelial transport of sodium ions (Na^+) by alveolar type II and possibly type I cells. The rate-limiting step in this process is the activity of the heterotrimeric apical membrane epithelial Na^+ channel (ENaC). Pharmacologic inhibitors and genetic manipulations that disrupt Na^+ transport result in fluid accumulation within the lung and failure of gas exchange. The importance of Na^+ transport in the lung is also demonstrated in conditions such as ARDS, where abnormal absorption of Na^+ contributes to the pathophysiology of pulmonary disease. ENaC expression and function is influenced by diverse factors, such as oxygen tension, glucocorticoids, and cytoskeletal proteins. In addition, ENaC dysfunction has been shown to be induced by purinergic nucleotide activation of P2Y receptors (in paramyxoviral bronchiolitis) and reactive species (in acute lung injury). Finally, β-adrenergic agonists have been shown experimentally to reverse defects in ENaC function, and improve hypoxemia and pulmonary edema, and may provide a novel therapeutic modality for ARDS, although some viral lung pathogens appear to induce insensitivity to their actions.

Key Words: respiratory virus**,** β-adrenergic agonist**,** P2Y receptor**,** protein kinase C

THE ROLE OF SODIUM CHANNELS IN LUNG PHYSIOLOGY

For gas exchange to occur, the epithelium of the lung must maintain a humidified atmosphere with only a thin layer of fluid lining the airway surface. Absorption of fluid out of the airway and alveolar lumen requires active transport of sodium ions (Na^+) from the apical surface of the pulmonary epithelium, across the apical and basolateral membranes of epithelial cells, and into the interstitial space and/or bloodstream. Pharmacologic inhibitors and genetic manipulations that disrupt Na^+ transport result in fluid accumulation within the lung and failure of gas exchange. The importance of Na^+ transport in the lung is also demonstrated in several human disease processes, where

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abnormal absorption of Na^+ contributes to the pathophysiology of pulmonary disease.

While type I alveolar pneumocytes line >95% of the distal lung surface, alveolar type II pneumocytes (ATII cells) may mediate most of the ion and fluid transport (52). ATII cells, which make up 67% of the total number of alveolar epithelial cells, can be isolated with high purity, and grown as confluent monolayers (19; 51). Electrophysiological studies of cultured ATII cells have identified apical plasma membrane cation channels, referred to as epithelial Na^+ channels (ENaC; reviewed in (53)). These channels have a higher permeability to Na^+ than other cations and can be blocked by the diuretic drug amiloride (4). Na^+ ions diffuse passively into ATII cells (and possibly ATI cells, which have been shown to express ENaC subunit proteins and to transport Na^+ ions in vitro (7; 38)) through these apical cation channels (36; 76; 76) and are extruded across the basolateral membranes by the ouabain-sensitive Na^+,K^+-ATPase (20). While the driving force for Na^+ transport is produced by the basolateral Na^+,K^+-ATPase, it is the apical entry of Na^+ ions through ENaC which is the rate-limiting step for Na^+ flux. Indeed, the apical plasma membrane ENaC channels offer more than 90% of the overall resistance to transepithelial Na^+ transport.

The expression and function of ENaC is highly regulated. Multiple hormones and signaling pathways influence not only expression of the channels, but also post-translational modifications that regulate channel function. By understanding Na^+ transport at the molecular level, we can better understand the molecular pathogenesis of lung disease and design more appropriate therapies and interventions.

Biology of ENaC in the lung

ENaC is a heterotrimer of 3 transmembrane subunits (α, β, and γ), which are expressed in unequal proportions in respiratory epithelia (12), although the exact stoichiometry remains controversial. Some studies have indicated that ENaC forms a tetrameric complex (2α, β, γ) (21), while others have provided data indicating that the ENaC channel is a much larger complex (3α, 3β, 3γ) (68). mRNA for all 3 subunits of ENaC is present in the lungs of both humans and mice (10), but the Na^+ channels identified to date in airway epithelia display variable biophysical characteristics (single-channel conductance, P_{Na}/P_k, and affinity for amiloride and its ethylisopropyl analog (53)). For example, expression of α, β, and γ ENaC in Xenopus oocytes is associated with formation of highly Na^+ selective cation channels (12), but in ATII cells both nonselective and highly Na^+ selective cation channels have been identified (76). It appears that αENaC is sufficient to form functional amiloride-sensitive NSC channels, but the presence of β and γ subunits significantly enhances channel activity and substantively changes gating characteristics of the channel to the HSC form (12; 34; 36).

ENaC channels are constitutively open at the plasma membrane and do not appear to require additional activation (12). However, factors that influence both ENaC mRNA and protein levels can potentially modulate amiloride-sensitive Na^+ transport in the lung. Second messengers and signaling molecules may be able to alter the open probability of ENaC either by direct modification (phosphorylation/dephosphorylation) or by altering protein-protein interactions. While ENaC expression and function is known to be influenced by diverse factors, such as oxygen tension (62), glucocorticoids (73), and cytoskeletal proteins (5; 64), this review will concentrate upon three systems which

we have found to be involved in the pathogenesis of ENaC dysfunction in adult lung disease: the purinergic nucleotide system, reactive species, and β-adrenergic/cAMP agonists.

ENaC in adult lung disease

Na^+ transport appears to be essential for maintenance of a normal gas diffusion distance in the adult lung. In adults with acute respiratory distress syndrome (ARDS), Matthay and Wiener-Kronish (54) found a positive correlation between the ability of the alveolar epithelium to transport Na^+ actively and the rate of resolution of noncardiogenic pulmonary edema. Similarly, instillation of the epithelial Na^+ channel blocker phenamil into the lungs of rats exposed to hyperoxia resulted in higher levels of extravascular lung fluid volumes 24 hours later (75). Interestingly, the venom of a South American scorpion (Tityus serrulatus), which causes fatal respiratory failure and pulmonary edema, also decreases lung liquid clearance, probably by downregulating Na^+,K^+-ATPase in the alveolar epithelium (13). Finally, patients with systemic pseudohypoaldosteronism, caused by loss of function mutations in the genes encoding ENaC subunits, completely lack electrogenic Na^+ transport in the upper and lower airways. In some cases, pseudohypoaldosteronism results in a doubling of ALF volume, persistent rhinorrhea, and recurrent respiratory illness (39).

Effect of pulmonary pathogens on Na^+ transport

Despite the fact that fluid and mucus accumulation in airways and lung tissue is a major component of most respiratory infections (48), the effect of pathogens on respiratory epithelial Na^+ transport has not been studied in detail. Several lung pathogens have been shown to inhibit Na^+ transport by respiratory epithelia *in vitro*. Mycoplasma pulmonis inhibits amiloride-sensitive Na^+ absorption and cholinergic-stimulated Cl^- secretion by C57BL/6 mouse tracheal epithelial cells (45). Similarly, Pseudomonas aeruginosa rhamnolipids inhibit amiloride-sensitive Na^+ transport by ovine tracheal epithelium (26), while the hemolysin blocks active Na^+ uptake and Cl^- secretion by canine bronchial epithelium (71). Mycobacterium tuberculosis (77), pneumotropic, but not neurotropic, influenza A virus (42), and Sendai virus (43) have also been shown to inhibit ENaC activity in vitro. Influenza A virus rapidly (within 60 minutes of infection) inhibits amiloride-sensitive Na^+ transport by mouse tracheal epithelial cells. This inhibition is mediated by binding of viral hemagglutinin to cell surface sialic acid moieties, and subsequent activation of phospholipase C and PKC.

Interestingly, the inhibitory effects of pathogens on ENaC found in vitro have not always been found in vivo. For example, in rats with P. aeruginosa pneumonia, alveolar fluid clearance (AFC), which is a functional index of ENaC function, increased 24 hours after infection, and this increase, which was inhibited by amiloride, was at least partially mediated by TNF-α (63). Similarly, instillation of Escherichia coli endotoxin into the lungs of rats resulted in a significant increase in AFC at 24 and 40 hours (25). Whether such increases in AFC have detrimental pathophysiologic consequences, or whether they are the result of sublethal injury to the alveolar epithelium resulting in its repopulation with increased numbers of ATII cells, remains to be determined. Nev-

ertheless, some pulmonary pathogens may in contrast induce hypoxemia as a result of inhibition of AFC. For example, we recently reported that replicating respiratory syncytial virus (RSV) reduces the AFC of the bronchoalveolar epithelium in vivo (Fig 1), without inducing detectable epithelial cell death or an increase in alveolar permeability to albumin (17). Interestingly, we found that RSV-mediated inhibition of AFC was not related to viral loads per se: instead it was mediated by uridine triphosphate (UTP), acting in autocrine fashion on P2YR on bronchoalveolar epithelial cells. Specifically, we found increased levels of UTP in the bronchoalveolar lavage fluid of Balb/c mice 2 days following RSV infection (Fig 2). Moreover, reduced AFC was associated with increased lung water content, and peripheral hypoxemia (16). Addition of apyrase, which degrades both ATP and UTP, or XAMR0721 (a P2Y inhibitor) in the instillate prevented the decrease of AFC (17). Finally, UTP instilled in the alveolar space of Balb/c mice decreased AFC (Fig 3). Reduced AFC may result in formation of an increased volume of fluid mucus, airway congestion, and rhinorrhea, all features of severe RSV disease.

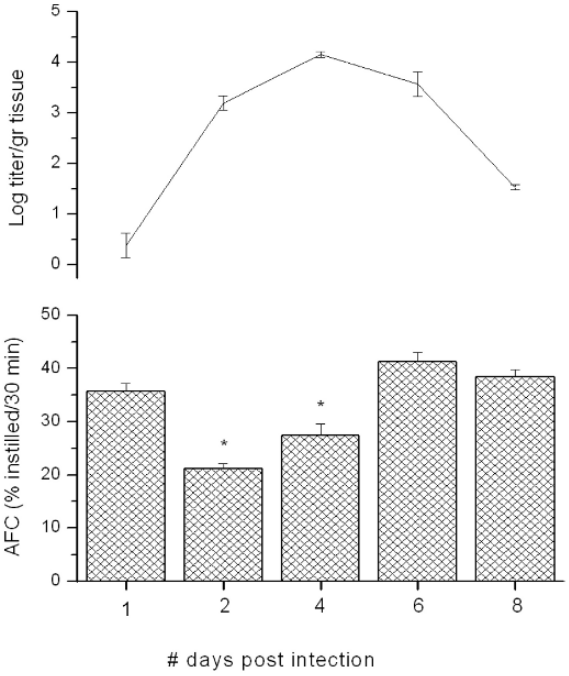

Figure 1. Intranasal infection of BALB/c mice with RSV results in significant inhibition of alveolar fluid clearance (AFC) at days 2 and 4 after infection. Mice were infectected with $2x10^6$ plaque forming units of RSV, suspended in 100 µl of buffer, intranasally as previously described (17). Alveolar fluid clearance (lower pannel; expressed as % of instilled fluid per 30 min) was measured across anesthetized, ventilated mice, with normal oxygenation and acid-base balance. n = 6-23 per group, as previously described (17). Mock infection had no effect on AFC. AFC was inhibited by 43% at day 2 and by 26% at day 4. RSV titers in lung tissues (upper pannel; expressed as the log of RSV PFUs per gr lung tissue) peaked at 4 days post infection and then decreased. Threshold of detection is 1 log. Notice the lack of correlation between RSV titers and AFC. Values are means ± SE. *$p<0.005$, compared with uninfected mice. Modified from ref 17.

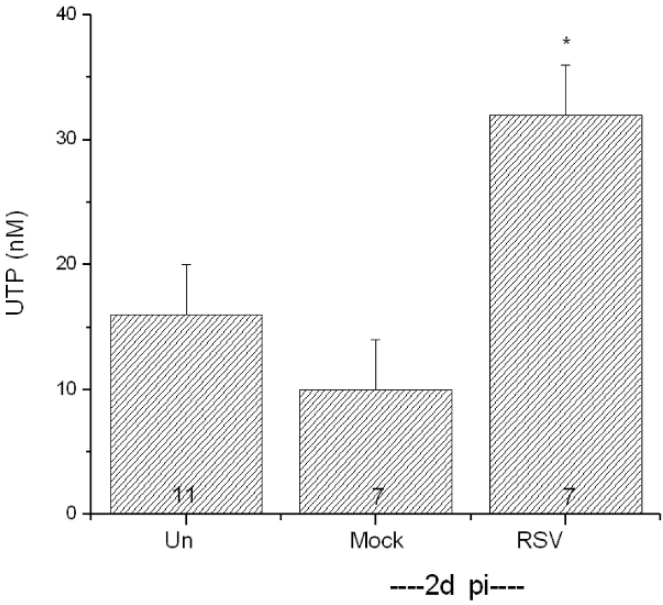

Figure. 2. RSV infection increases UTP levels in the BAL of Balb/c mice. Mice were infected with RSV as described in the legend of Figure 1. After 2 days, they were sacrificed and their lungs were lavaged. Endogenous nucleotidases in BAL fluid were heat denatured (100°C, 3 minutes) and UTP content was measured using the UDP-glucose pyrophosphorylase as previously described (16). Numbers are means ±1 SEM; * $<p<0.005$ compared to either unistilled or mock instilled mice. Labels as follows: Un = Uninstilled mice; Mock = Mock infected mice; RSV = infected with 2 x 10^6 plaque forming units of RSV. Numbers in bars indicate numbers of mice in each group.

ENaC modulation by purinergic nucleotides

Purinergic 5'-nucleotides are known modulators of ENaC activity in respiratory epithelial cells. Relatively large amounts of adenosine triphosphate (ATP) and UTP are released by human respiratory epithelial cells in vitro, although the underlying pathway for nucleotide release remains undefined (30; 46). Released ATP is rapidly metabolized to a mixture of ADP, AMP, and adenosine. ATP, its metabolites, and UTP each have inhibitory effects on ENaC, mediated via interaction with purinergic receptors expressed on respiratory epithelial cells (11; 40). Both ATP and UTP are known to inhibit respiratory epithelial Na^+ absorption in vitro (32; 33; 35; 50), via interaction with P2Y purinoceptors (P2YR). In vivo, UTP administered at pharmacologic doses (100 μM) to human subjects has also been shown to induce Cl^- secretion by nasal epithelium (41) and, when given by aerosol, to promote mucociliary clearance, although, interestingly, in the presence of amiloride, it also induced mild hypoxemia (60).

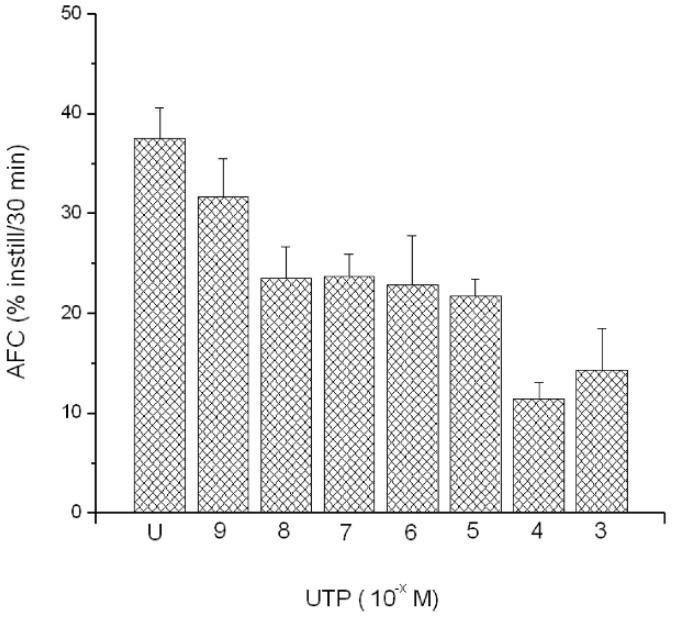

Figure 3. Instillation of UTP in the alveolar space of Balb/c mice decreases AFC. To confirm that UTP alone can recapitulate the inhibitory effects of RSV on alveolar fluid clearance, we instilled 5% BSA (the standard instillate for the measurement of AFC) containing ten-fold dilutions of UTP into the lungs of normal mice and determined AFC 30 min later. Shown values are means ± 1 SEM (n 3-4 per group). Final doses of UTP (from 1mM to 10 nM) had a significant inhibitory effect on AFC, but 1 nM UTP had no effect. 1 mM and 100 μM UTP induced significantly greater inhibition of AFC than that caused by RSV (62% and 70%, respectively), while doses from 1 μM to 10 nM caused inhibition similar to that induced by infection with RSV for 2 or 4 days (42-36%). Modified from ref. 17.

Protein kinase C as a central regulator of ENaC function?

Until recently, the mechanism by which nucleotide binding to P2YR might induce reduced ENaC activity has remained unclear. While downstream signaling events mediating ENaC downregulation have not yet been fully defined, it is known that P2YR are G-protein-coupled, and act via the inositol phosphate pathway to stimulate calcium release from intracellular stores, but can also act via multiple secondary signal transduction pathways including protein kinase C (PKC) (11). Activation of PKC has been shown to reduce ENaC activity and modify its subunit composition, although the isoforms of PKC involved have not been defined. Inhibition of PKC rapidly increased P_o and appearance of new channels in patches of A6 cells (47). In contrast, stimulation of PKC inhibited whole-cell currents in Xenopus oocytes (2). Likewise, PKC activation decreased expression of both β and γ, but not α ENaC subunit proteins in A6 cells by 3h and 14h, respectively, and also resulted in a decrease in transepithelial Na^+ reabsorption (70).

Recent data indicates that P2YR-mediated ENaC downregulation may also involve activation of the ubiquitin-proteasome pathway, which is an important regulator of ENaC function. The half-life of ENaC in mammalian cell membranes is short (less than 1 hour). ENaC is ubiquitinated in vivo on the α and γ, but not β subunits (69). Inhibition of ubiquitination or the proteasome results in increased channel activity, due to an increase in the number of channels present at the plasma membrane (49). Ubiquitination (ATP-dependent serial addition of ubiquitin monomers to lysine residues on proteins), which targets proteins for rapid degradation by the proteasome, is catalyzed by the sequential action of ubiquitin-activating, ubiquitin-conjugating, and ubiquitin protein ligase enzymes (29). Neural precursor cell-expressed developmentally downregulated protein 4 (Nedd4) is the ubiquitin-protein ligase required for ubiquitination of ENaC (69). Nedd4 directly regulates basal ENaC activity by modulating channel stability at the cell surface. In the lung, Nedd4 is mainly expressed in the epithelia lining the airways and in the distal respiratory epithelium, a pattern of expression similar to that of ENaC (8). Interestingly, the interaction between ENaC and Nedd4 is disrupted in Liddle Syndrome, a hereditable form of salt-sensitive hypertension (1). Liddle syndrome mutations in the βENaC cytoplasmic domain disrupt the association of Nedd4 with the C-terminus of βENaC. As a result, ENaC has a longer half-life in the plasma membrane and is less efficiently internalized and degraded. This leads to increased amiloride-sensitive current at the apical membrane and increased salt absorption.

The link between PKC and the ubiquitin-proteasome pathway has just recently been made clear. In A6 cells, PKC has been shown to activate the mitogen-activated protein (MAP) kinase Raf-1, and the MAP kinase kinases MAPK/ERK (MEK) 1 and 2. Activation of MEK 1 and 2 was shown to enhance phosphorylation of β and γ, but not α ENaC (67). This phosphorylation event facilitates binding of Nedd4-2 to ENaC, which may then promote ENaC internalization and removal from the cell surface (69). Therefore, purinergic stimulation and PKC activation may decrease ENaC function both through altered ENaC phosphorylation and altered ENaC degradation.

Inflammatory mediators of ENaC dysfunction in pulmonary disease

Reactive oxygen/nitrogen species (RONS), such as the free radicals nitric oxide (•NO) and nitric dioxide ($•NO_2$) as well as peroxynitrite anions ($ONOO^-$), are known to inhibit the activity of both ENaC (31) and the ATII cell Na^+/K^+ ATPase (72) in vitro, via both cGMP-dependent and cGMP-independent mechanisms. In pulmonary inflammatory disease, increased levels of RONS may directly modify ion transporters, disrupt their association with chaperone or structural proteins (such as actin), or alter signal transduction pathways, all of which may result in impaired Na^+ absorption across the alveolar epithelium. Nitrotyrosine (the stable by-product of $•NO_2$ reaction with tyrosine residues in proteins (3)) has been detected in the lungs of patients with acute lung injury (44) and those with hantavirus cardiopulmonary syndrome (HCPS) (18). Likewise, both nitrotyrosine and large amounts of nitrate, the stable by-product of peroxynitrite and nitrogen dioxide, have been found in the lavage fluids of patients with acute lung injury and the plasma of hantavirus cardiopulmonary syndrome cases (18). These find-

ings indicate that reactive oxygen-nitrogen species are produced in the lungs of patients with inflammatory disease, and may contribute to its pathogenesis. In a recent study, Modelska et al. (56) showed that absorption of isotonic fluid, secondary to Na^+ absorption across the alveolar space, was inhibited followed prolonged hemorrhagic shock. Moreover, instillation of aminoguanidine, a nitric oxide synthase inhibitor, restored fluid absorption to normal levels. Thus, increased production of reactive oxygen-nitrogen species by lung epithelial or inflammatory cells may modify molecules required for Na^+ transport across the alveolar epithelium.

Finally, it should also be noted that certain proinflammatory cytokines have also been shown to directly alter Na^+ transport by respiratory epithelial cells in vitro. Specifically, Na^+ transport is inhibited by IFN-γ (23), and IL-4 (24), while TNF-α has been shown to both increase (22; 63) and reduce (15) Na^+ transport, in vivo.

Effect of β-adrenergic agonists on ENaC function

β-adrenergic receptor agonists (β-agonists) have been shown to improve AFC in animal models of lung injury in which AFC is impaired, by increasing the activity of both epithelial Na^+ channels and Na^+, K^+ ATPase (reviewed in (58)). β-agonist prophylaxis has also been shown to be of value in reducing the incidence of high altitude pulmonary edema (itself a consequence of impaired AFC secondary to hypoxia at high altitude) in susceptible mountaineers (66), and intravenous salbutamol treatment can reduce extravascular lung water in patients with acute lung injury (61). Because of such encouraging findings, the National Heart, Lung, and Blood Institute Acute Respiratory Distress Syndrome (ARDS) Network is planning to conduct a large, multicenter, prospective clinical trial to test the potential efficacy of the aerosolized β-agonist albuterol in ventilated patients with ARDS (74).

Stimulatory effects of β-agonists on AFC have been shown to be mediated via activation of adenylyl cyclase, which generates cAMP and thereby stimulates cAMP-dependent protein kinase production (protein kinase A; reviewed in (37)). PKA phosphorylates cytoskeletal proteins and promotes both exocytosis to the cell membrane and direct phosphorylation of ENaC β and γ subunits (5). β-agonists also increase the expression of ENaC α-subunit mRNA and protein (55), phosphorylation-dependent translocation of Na^+, K^+ ATPase pumps from intracellular pools to the basolateral membrane of epithelial cells (6), and apical Cl^- flux through the cystic fibrosis transmembrane conductance regulator (CFTR) (59). However, while it appears that functional β-AR are essential for adaptation to pulmonary edema, it remains unclear whether they are required for maintenance of alveolar fluid balance in the normal lung (58).

Interestingly, several respiratory tract viral pathogens have been shown to modulate β-adrenergic receptor agonist function. Tracheal smooth muscle from influenza virus-infected mice has reduced sensitivity to β-agonists and forskolin ex vivo (28), and airway segments taken from ovalbumin-sensitized guinea pigs exhibit impaired bronchodilator responses following infection with parainfluenza 3 virus (9). Likewise, human airway smooth muscle tissue exhibits reduced responsiveness to β-agonists, associated with increased $G\alpha_i$ expression, following in vitro infection with rhinovirus (27) and RSV (57). Finally, we have recently found that RSV induces heterologous desensitiza-

tion of bronchoalveolar epithelial β-AR in vivo (Ian Davis, unpublished observations). These findings have important implications for dysregulation of other β-agonist-mediated responses in respiratory epithelium and airway smooth muscle following viral infection: β-agonists are known to promote mucociliary clearance and fluid secretion by submucosal glands, may have anti-inflammatory effects, and are widely used as bronchodilators (reviewed in (65)).

CONCLUSIONS

Na^+ transport in the distal lung is required for normal lung function. Defective Na^+ absorption may contribute to the pathogenesis of acute and chronic lung disease. As the molecular mechanisms of lung injury and disease are better characterized, we may better understand the contribution of ENaC function to pulmonary disease. The demonstration that RONS released by activated alveolar macrophages down regulate the activity of alveolar epithelial cell Na^+ channels (14) provides an example of how mediators of lung injury could influence Na^+ absorption during the progression of pneumonia or ARDS. Manipulating ENaC function may provide new treatments for both ARDS and pulmonary infectious diseases. For example, strategies that inhibit UTP-P2YR interaction can now be evaluated as therapeutics for RSV pneumonitis, a condition for which specific antiviral drugs are sadly lacking. Identification of additional targets for regulating ENaC expression and function in the lung may provide further opportunities for clinicians to target Na^+ absorption in the lung. Moreover, by studying Na^+ channel physiology in the context of human lung disease, we may learn more about the basic physiology of distal lung transport.

ABBREVIATIONS

AFC = alveolar fluid clearance; ATII = alveolar type II cells; ENaC = epithelial sodium channels; RSV = respiratory syncytial virus; UTP = urine triphosphate; RONS = reactive oxygen-nitrogen

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Chapter 11

LUNG INTERSTITIAL PRESSURE AND STRUCTURE IN ACUTE HYPOXIA

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Abstract: The air blood barrier is a gas exchanger and is well designed to fulfill this task as its main feature is its minimum thickness that in turn reflects a minimum amount of extravascular water. The maintenance of a minimum water volume is due to mechanisms able to control interstitial fluid turnover and to offset transient conditions of increase in this volume. The hydraulic pressure in the lung interstitium is ~ -10 cmH_2O and reflects the equilibrium between the lymphatic absorption pressure and the microvascular filtration through the basement membrane whose hydraulic permeability is kept very low due to the macromolecular organization of heparansulphate proteoglycans (HS-PGs). When microvascular filtration is increased, the increase in extravascular water is minimal in face of a considerable increase in interstitial pressure (up to ~ 5 cmH_2O) because of the high elastance of the extracellular matrix thanks to the mechanical role of matrix chondroitin sulphate proteoglycans (CS-PGs). This increase in pressure buffers microvascular filtration. Hypoxia causes fragmentation of CS-PGs of the extracellular matrix and of HS-PGs of the basement membrane: the result is a decrease in tissue elastance and an increase in permeability of the endothelial and epithelial barriers. When the overall PGs fragmentation overcomes a critical threshold, severe lung edema develops. Recovery from severe lung edema requires that extracellular integrity is restored. We provide evidence for a prompt lung cellular response to interstitial edema. We interpret this response as a fine mechanism to detect minor increases in extravascular water and to promote the reparative process.

Key Words: proteoglycans, tissue elastance, mechanotransduction, lipid microdomains

INTRODUCTION

Control of extravascular water differs among organs and body compartments reflecting functional conditions. In some regions the volume of extravascular water is kept at a minimum as in the case of serous spaces (pleural cavity, abdominal cavity, joints), in the brain and in the lung. In the lung a minimum amount of extravascular water assures a minimum thickness of the air-blood barrier to favour gas diffusion.

The maintenance of a minimum water volume is due to mechanisms able to control interstitial fluid turnover and to offset transient conditions of increase in this volume. In

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this article we will review the mechanisms controlling extravascular water volume in the lung in response to hypoxia and how failure of these mechanisms lead to development of edema, a severe complication of high altitude climbing (27).

The exposure to hypoxia involves various adaptive responses from cellular to organ level and is aimed to defend oxygen availability to tissues. At cellular level it is known that cells sense oxygen concentration and respond to reduced O_2 availability mainly through the transcriptional regulator hypoxia-inducible factor (HIF-1) (28). At systemic level chemoreceptor stimulation triggers an increase in pulmonary ventilation and cardiac output.

MACROMOLECULAR ORGANISATION OF THE EXTRAVASCULAR SPACE

The lungs act as a gas exchanger and the air blood barrier is well designed to fulfil this task. As shown in Fig.1 the air-blood barrier (ABB) includes the "thin" and the "thick" portion. The "thin portion" is made simply of the endothelium, the epithelium (covered by a thin layer of water and surfactant) and an intervening fused basement membrane, it accounts for almost 50% of ABB and is the preferential site for gas exchange as the three layers provide a thickness of only 0.2-0.3 μm (2); the "thick portion" of the ABB contains the fibrillar structure and lymphatics. One can identify two functions for the macromolecular organization of the extracellular matrix. The fibrillar component mostly present in the "thick portion", including collagen I and III and elastic fibers, provides the elasticity of the lung tissue needed for continuous stretching and de-stretching. Another macromolecular component, that includes hyaluronan (HA) and proteoglycans (PGs) (25), fills the voids among the fibrillar structures and the cells, keeps the various structures assembled allowing however their reciprocal movements; furthermore it controls the permeability of the pores or channels through which water circulates. Indeed HA and PGs, being highly hydrophilic, can bind water to form gel-like structures producing two effects: a decrease in the volume of water in the free liquid phase and a reduction in the size of the pores or channels through which free water circulates. In fact, the hydration level of HA and PGs represents an efficient system to control permselectivity of the basement membrane. Fig.2 is a schematic drawing depicting the macromolecular components of the interstitial matrix. HA is a glycosaminoglycan that can reach a very high molecular weight (10^6 daltons) (10, 25) due to multiple repetitions of disaccharides made by an uronic acid residue covalently linked to an N-acetyl-glucosamine; HA can be thought as a long molecule randomly winding as a coil in the extravascular space. PGs represent multidomain core proteins that contain one or more covalently linked glycosaminoglycan chains. Different PG populations are present in the lung parenchyma (25). Versican is a chondroitin sulphate PGs (CS-PG) of molecular mass > 1000 kDa present in the extracellular matrix, mostly in the alveolar interstitial spaces in regions not occupied by fibrillar components (25). Perlecan and agrin are important heparan sulphate PGs (HS-PG) of intermediate molecular weight (~ 300-500 kDa) present in the basement membrane, while syndecans

(< 300 kDa) are present at cell surface (3, 25, 33). HS-PGs are important homeostatic mediators acting at cell surface and contribute to cell-matrix adhesion and permselectivity (3, 10, 25). Decorin is a small dermatan sulphate proteoglycan (DS-PG) playing a role in the structural organization of the collagen fibrils (25).

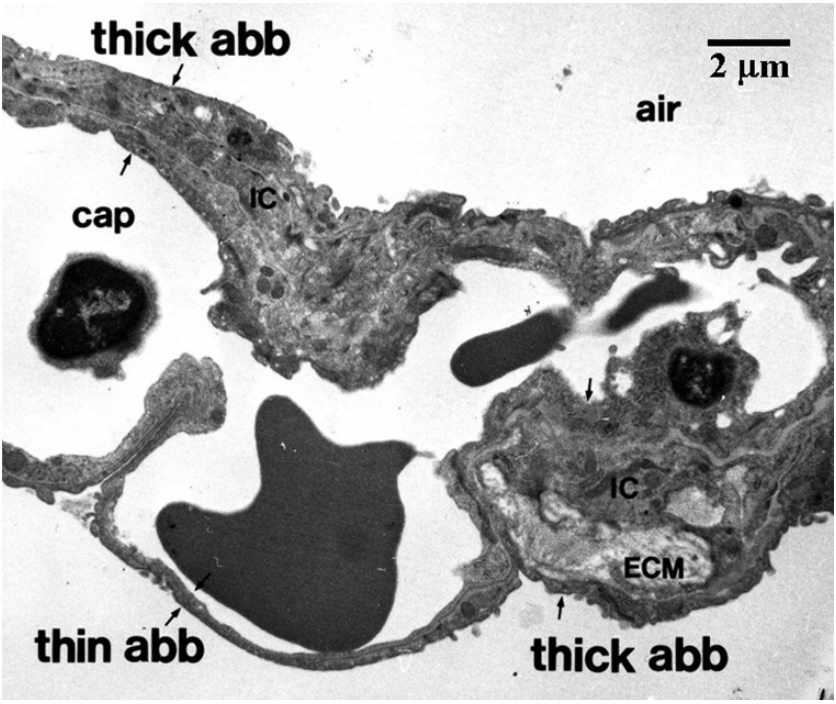

Figure 1. Microphotograph of the air blood barrier (abb) , showing the thin and the thick portion. Cap: capillary; IC: interstitial cells; ECM: extracellular matrix.

Versican molecules are important for the mechanical integrity of the extracellular matrix due to their multiple co-valent bindings to the random coiled HA molecule through specific link proteins. Fort the rest, most of the PGs organization in the extracellular matrix is based on non-covalent linkages with other macromolecules, involving low-energy ionic and/or hydrophobic interactions that allow a certain mobility of the structures. A peculiar physical property of hydrated PGs is their resistance to compressive forces. PGs play a pivotal role in tissue development and repair by interacting with inflammatory cells, proteases and growth factors (9, 25). The structural integrity of the pulmonary interstitium depends upon the balance between synthesis and degradation.

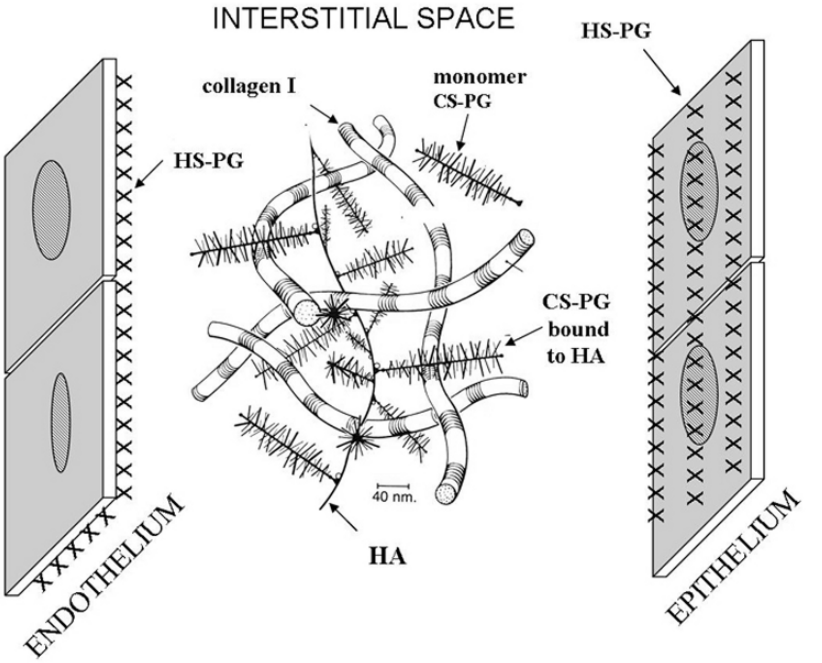

Figure 2. Schema showing the various components of the macromolecular organisation of the lung interstitial space. HA: hyaluronan; HS-PG: heparansulphate proteoglycans of the basement membrane; CS-PG: chondroitinsulphate proteoglycans in the interfibrillar substance.

LUNG FLUID BALANCE IN PHYSIOLOGICAL CONDITIONS

According to the revisited Starling law, the bulk flow of water across the endothelium depends upon the resultant between hydraulic and colloid osmotic pressures (P and Π, respectively) between capillaries (subscript *c*) and interstitial space (subscript *i*):

$$1. \quad J_f = L_p \cdot S[(P_c - P_i) - \sigma (\Pi_c - \Pi_i)]$$

where *Lp* is the coefficient of hydraulic permeability, *S* is the surface available for microvascular exchanges and σ is the solvent drag reflection coefficient of the endothelium for total plasma proteins.

A key variable in eq. 1 is the hydraulic pressure of the free water phase in the interstitial compartment, *Pi,* that represents the resultant between microvascular filtration and lymphatic drainage. We developed the "pleural window" technique (17) that al-

lowed us to directly measure *Pi* by micropuncture keeping the lungs physiologically expanded in the chest wall (Fig. 3). The same technique allowed us to derive indications on the changes of extravascular water volume from the geometry of the perivascular interstitial space (30). Under physiological conditions (Fig. 4) *Pi* is ~ -10 cmH_2O and a Starling pressure gradient (as from eq. 1) of about 10 cm H_2O causes microvascular filtration (17). The subatmospheric value of *Pi*, indicating a rather dehydrated condition of the lung interstitium, reflects two important features: the ability of lymphatics to generate a subatmospheric pressure (19), and a low microvascular filtration due to a low hydraulic permeability (*Lp*) (29). The value of σ averages 0.7 in physiological conditions (29). Note that microvascular filtration in the lung occurs through an astonishingly high capillary surface area (*S* in eq. 1) estimated at 2500 cm^2/g of tissue. Under physiological conditions, microvascular filtration is matched by lymphatic absorption.

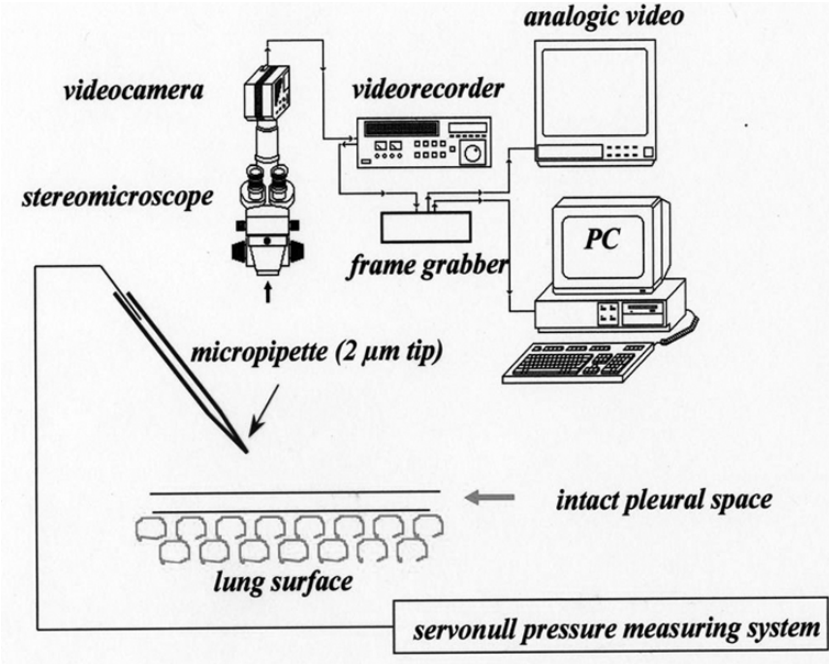

Figure 3. Assembly of the equipment for transpleural micropuncture of the lung kept intact in the pleural space.

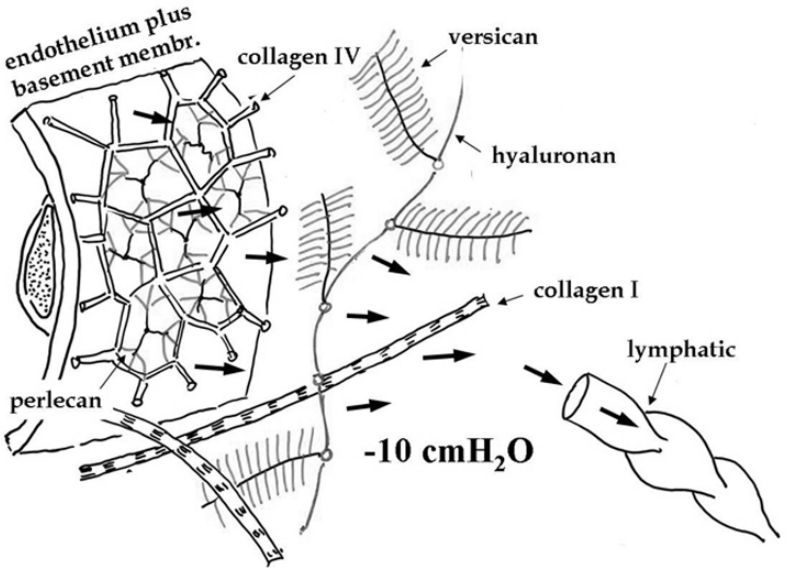

Figure 4. Schematic representation of the fluid turnover in the lung interstitium in physiological conditions. The subatmospheric interstitial pressure results from the balance between the absorption pressure of lymphatics and the microvascular filtration that is very low due to the low hydraulic permeability of the endothelial barrier. In control conditions filtration is matched by lymphatic drainage.

FROM THE PHYSIOLOGICAL CONDITION TO INTERSTITIAL LUNG EDEMA

We evaluated the perturbations induced on lung fluid balance in anesthetized rabbits exposed to 12% O_2, up to 6 h, a degree of hypoxia comparable to an altitude exposure of about 6000 m (16). Pulmonary artery pressure roughly doubled at 3h of hypoxia (from 18 ± 5 to 32±5mmHg) while left atrial pressure increased from 5 to 8 cmH O. Arterial PO_2, PCO_2 and pH remained essentially steady during hypoxia exposure and were 38 16 mmHg, 26 ± 5 and 7.38 ± 0.11, respectively.

As Fig. 5 shows, despite an increase in pulmonary artery pressure, no real change was found in capillary pressure (*Pc* in eq. 1) due to a remarkable increase in arteriolar precapillary resistance induced by edema (22). Exposure to hypoxia led to capillary recruitment (4, 31) and therefore to an increase in surface area (*S* in eq.1) for microvascular filtration. Furthermore, hypoxia is also a known cause of increase in *Lp* (8, 24). We like to emphasize that in eq.1 *Pc* is an additive factor for the Starling pressure gradient, while *S·Lp* is a multiplicative factor: therefore any increase in *Pc* is less important than an increase in *S·Lp*. In other words, doubling of *Pc* does not result in doubling of filtration rate, conversely, factor *S·Lp* can increase by more than one order of magnitude (24). Hypoxia also causes a decrease in σ, which implies that plasma proteins can be dragged more easily by water through the capillary endothelium (7).

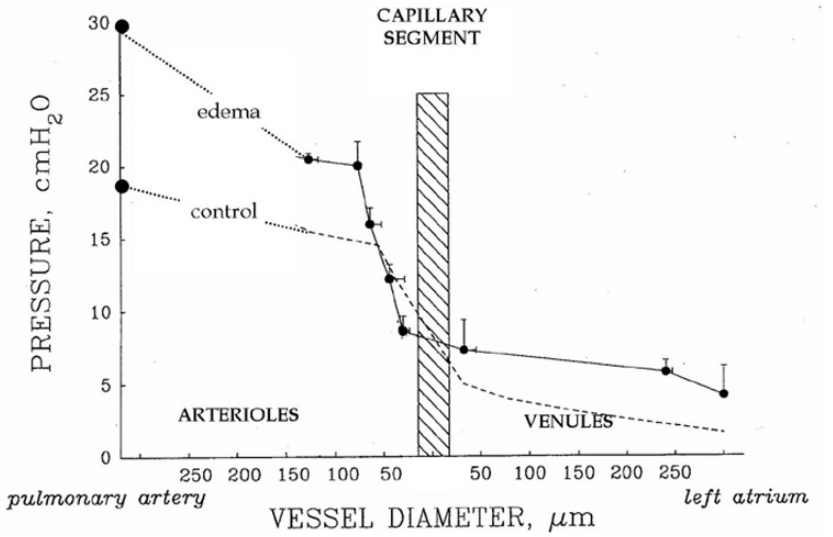

Figure 5. Microvascular pressure profile in physiological conditions and in interstitial edema.

As shown in Fig. 6, the increased microvascular filtration caused by hypoxia leads to an increase in pulmonary interstitial pressure up to ~ 5 cmH_2O. The ratio of the increase in interstitial pressure to the increase in volume of the extravascular water (not exceeding 10% in interstitial edema) is an estimate of lung tissue elastance and amounts to ~2 mmHg/(ml· 100 g wet tissue), a value about 20 fold higher compared to other organs (18). This mechanical response is also referred to as "tissue safety factor" against the development of lung edema because the increase in *Pi* reduces and, possibly, nullifies the filtration pressure gradient. The low tissue elastance reflects, in turn, the mechanical resistance of the macromolecular components of the extracellular matrix to the increased parenchymal stresses.

We analyzed the impact of hypoxia exposure on composition, structure and interaction properties of the proteoglycan component of the extracellular matrix. PGs were extracted from lung tissue specimen and their molecular size distribution was analyzed by gel filtration chromatography (16). The various peaks in Fig. 7 correspond to PG families of different size and composition. In normoxic lungs (top panel) large CS-PG (Mr > 0.5 MDa) accounted for ~ 25 % of total extracted PGs; HS-PG (0.5 - 0.1 MDa) for ~ 35 % and finally peptidoglycans (PDGL; < 100,000 Da) for 40 %. After hypoxia exposure (3h and 6h, middle and lower panels, respectively), the relative content of CS-PG and HS-PG was progressively reduced. The interpretation of the modification in the elution patterns is that hypoxia progressively caused fragmentation of CS-PG and HS-PG families and fragments were recovered in the PDGL fraction, which indeed increased relative to the other fractions. Therefore, hypoxia leads to a progressive damage of the proteoglycans mesh and this stems for the "inflammatory" nature of the hypoxia induced lung edema. On the other hand, this same feature is shared by lung edema induced by saline infusion as it entails a marked increase in TNFα mRNA (26).

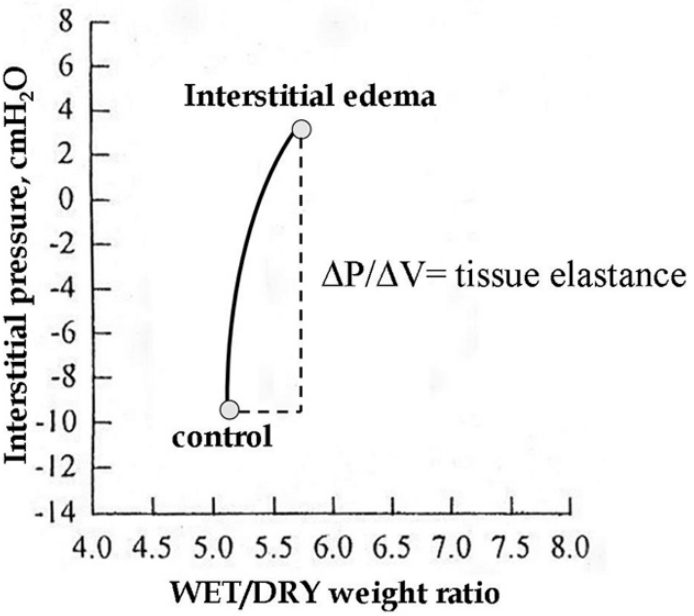

Figure 6. The increase in pulmonary interstitial pressure during development of interstitial edema. The ratio of the increase in pressure to the increase in extravascular water yields the elastance of the lung tissue.

FROM INTERSTITIAL TO SEVERE LUNG EDEMA

In general, the lung seems to be well designed to resist to edema formation. In fact, due to the high elastance of the lung tissue, a condition of interstitial edema (Fig. 8B) implies a considerable increase in parenchymal stresses but a negligible increase in extravascular water relative to control (Fig. 8A): accordingly, the air-blood barrier retains its thinness and lung can still efficiently work as a gas exchanger. Nevertheless, a sustained condition of interstitial edema is a cause of progressive loss of integrity of the interstitial matrix; in fact, we found that an increasing amount of hexuronate, an important component of proteoglycans families, is recovered from the lung tissue using a weak extraction agent, indicating a progressive loosening of the intermolecular bonds of PGs with the other components of the extracellular matrix (16). The obvious question then is: what is the mechanical resistance of the matrix macromolecules to a continuous increased stress?

Our experimental evidence is that the lung can resist for hours in a condition of interstitial edema (Fig. 8B) but the transition to severe edema (Fig. 8C) is a matter of a few minutes (20). This suggests that severe edema acutely develops when the damage to the extracellular matrix overcomes a critical threshold. In this respect, one should consider that normobaric hypoxia only leads to interstitial lung edema, and in fact severe edema could only be obtained by the saline infusion model. Due to the similarity in the process of interstitial matrix degradation caused by either hypoxia exposure or saline infusion we provide a common pathophysiological interpretation for

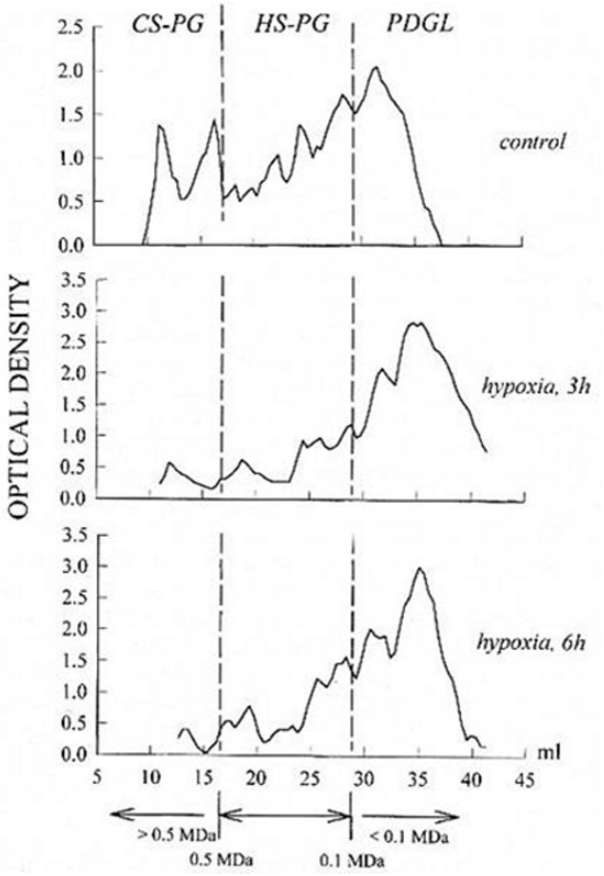

Figure 7. Gel-filtration chromatography of PGs extracted with 0.4 M GuHCl in control normoxia (top panel), after 3 hours (middle panel) and 6 hours of 12 % O2 (bottom panel). CS-PG, HS-PG and PDGL refer to chondroitin-sulphate, heparan-sulphate proteoglycans and to peptido-glycans, respectively. The two vertical dashed lines delimit the ranges of molecular weight for the three PG families.

the development of severe edema. Factors contributing to progressive disorganization of the proteoglycan mesh include: a) mechanical yielding, b) the increased distance, due to increased hydration, at sites of the non-covalent bonds of PGs with other matrix components (estimated of the order of 30 nm, (2), c) the activation of tissue metalloproteases MMP-2 and MMP-9 (16). Furthermore, the fragments of proteoglycans extracted from the edematous lung changed their native chemical properties, as reflected by a marked decrease in their binding properties to other macromolecules of the matrix (16, 20). These modifications clearly impact on the time course of pulmonary interstitial pressure: as shown in Fig. 9, interstitial pressure drops towards zero in the transition from interstitial to severe lung edema (20). This, in turn, restores a filtration pressure gradient and Fig. 9 shows that interstitial pressure remains unchanged in face of a marked increase in extravascular water. As can be appreciated from Fig. 8C, most of the increase in extravascular lung water in severe edema is due to alveolar flooding rather than to interstitial accumulation. Therefore, the time course of interstitial pressure in Fig. 9 can be accounted for by two coexisting mechanisms. First: the lung tissue

does not offer mechanical resistance to the increase in interstitial water due to the loss of integrity of the macromolecular extracellular matrix; in fact, the fragmentation of CS-PGs is a critical factor accounting for the loss of the "tissue safety factor". Second: the fragmentation of HS-PGs of the basement membrane likely increases the hydraulic permeability of both the endothelial and the epithelial barriers, which would reduce flow resistance for water to reach the alveoli, as schematically depicted in the insert of Fig. 9. This aspect was never considered before and in fact it should be regarded as a "protective" mechanism against the complete disassembly of the macromolecular interstitial structure. Hypoxia also causes a decrease in σ that allows plasma proteins to be dragged into the alveoli (as indicated by the pink staining of the alveolar fluid in Fig. 8C). It should be remembered that the increase in permeability leading to alveolar flooding may occurs through the paracellular route down intercellular openings of the order of 50-100 nm; therefore, the development of hypoxic lung edema does not require breaking or burst of pulmonary capillaries (32).

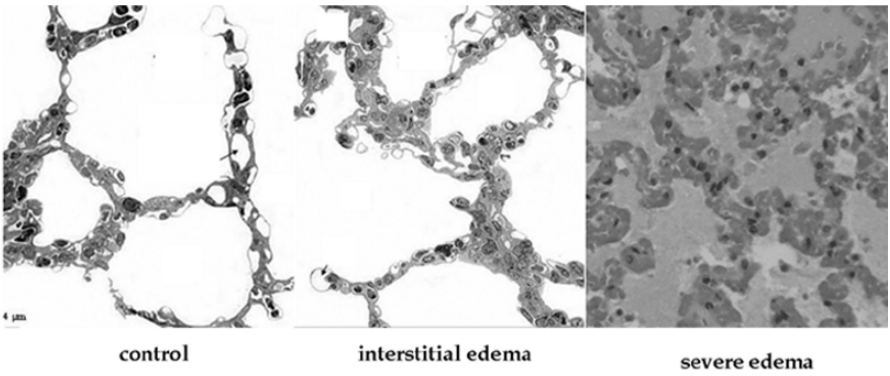

Figure 8. Conventional light microscopy images of rabbit lungs fixed in situ to allow a comparison between control normoxia, hypoxic interstitial edema and severe edema.

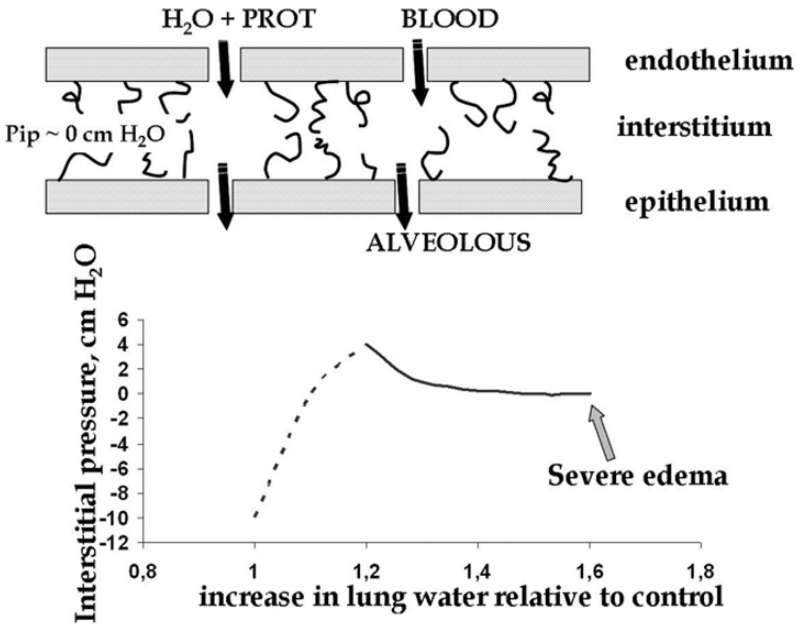

Figure 9. Lower panel: interstitial pressure drops to about zero as lung water increases during development of severe edema. Upper panel: fragmentation of PGs causes a decrease of tissue elastance to zero and also an increase in permeability of the endothelial and of the epithelial layer. Water and proteins leak into the alveoli with no increase in pulmonary interstitial pressure.

RECOVERY FROM LUNG EDEMA

As suggested above, a correlation exists between the development of severe edema and a massive fragmentation of the extracellular matrix, therefore, it appears logic to consider that recovery from edema, requires matrix remodelling. We did not develop an experimental model of recovery from hypoxic lung edema. Nevertheless, we can provide a good example to show that alveolar clearance requires an intact matrix: this is the case of absorption of the alveolar fluid in term newborn at birth. As shown in Fig. 10, the active epithelial water absorption from alveoli to the interstitial space causes an increase in interstitial pressure that peaks at about 6 cmH_2O at 2h of postnatal life (experimental study in newborn rabbits, (15)): this mechanical event reflects the low elastance of the interstitial tissue and is critical to generate a Starling pressure gradient for water transport into the pulmonary capillaries (as suggested by the insert) (15). With increasing time, interstitial pressure tends to decrease as more fluid is being drained in blood than is being absorbed into the interstitium; complete clearance of alveolar fluid in term newborn is a fairly rapid phenomenon, being completed in about 3-4 hs (15). In fact, the immature matrix structure is a cofactor that greatly impairs the alveolar clearance in premature newborn (14). Recovery from high-altitude lung edema is also fairly rapid, provided hypoxia, a major factor inhibiting protein synthe-

sis, is eliminated. Therefore, the suggestion is that remodelling of the matrix may be a rapid phenomenon.

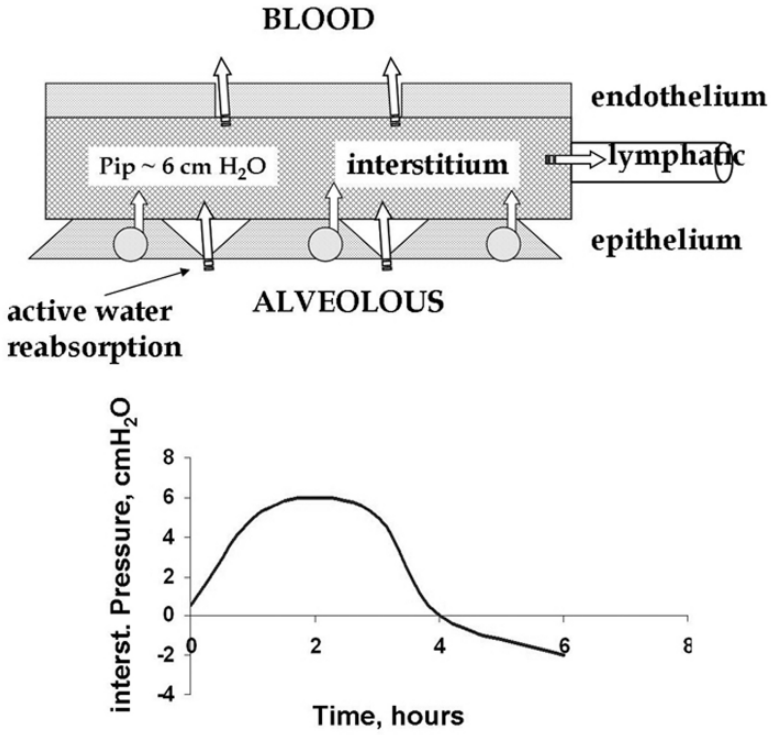

Figure 10. Bottom panel. An example of recovery from edema: the absorption of alveolar fluid in term newborn (data from anesthetized newborn rabbits). Active water absorption from the alveoli to the interstitial space increases interstitial pressure up to about 6 cm H2O, generating a Starling pressure gradient for water flow to the pulmonary capillaries. Upper panel: schema to show water transport from alveoli to pulmonary capillaries with intact interstitial matrix.

LUNG CELLULAR RESPONSE TO INTERSTITIAL LUNG EDEMA

We have studied the response of lung cells to the condition of interstitial edema that is on the edge between tissue lesion and repair. Lung epithelial and endothelial cells are in a position to act as good sensors since they are directly exposed to different potential stimuli originating at the interstitial, vascular or alveolar level. We reasoned that these cells might act as sensors of changing interstitial fluid dynamic conditions reasoning that the sequence of interstitial macromolecules fragmentation (16) might trigger signalling-transduction mechanisms to induce matrix remodelling (26). We

therefore evaluated the change in the expression of lipid microdomains on the plasma membrane surface. Lipid microdomains represent specific platforms for signal - transduction (6) and cover 10-15% of total plasma membrane surface. They may be present as flat regions, called lipid rafts, or as flask-like vesicles of about 70nm in diameter, named caveolae. We also estimated the morphological modifications in cell volume/ surface that represent a mechanism of signal transduction (5). Indeed, cells operate a fine tuning of volume/surface by the activation of mechanosensitive ion channels that function as transducers for forces generated at the cellular surface. As shown by the micrographs at x 66000 in Fig.11, endothelial and epithelial cells are extremely thin in physiological condition, the frequency distribution of their cytoplasm volume being remarkably skew, with excess values in the lowest range of volumes. At this point, it is of interest to compare the cellular modifications induced by interstitial lung edema caused by saline infusion (a model of cardiogenic lung edema, (4) or hypoxia exposure (1). Hypoxia caused a further thinning of the endothelial and epithelial cells, while cardiogenic edema caused opposite effects (1).

Fig. 12 shows that the number of caveolae was decreased in hypoxia and increased in cardiogenic edema (see also Fig. 11), as confirmed also by the increased expression of Cav-1, the protein marker of caveolae (1). Conversely, lipid rafts were increased in hypoxia and decreased in cardiogenic edema, as confirmed by the expression of their specific marker CD-55 (1).

Therefore, these data suggest a differential activation of signalling-transduction mechanisms in lung cells in response to interstitial edema as evidenced by the change in expression in lipid microdomains and in cellular volume/ surface control. We could also document a correlation between changes in plasma membrane composition and "membrane fluidity" as determined by the anisotropy parameter (23): membrane fluidity was decreased in hypoxic edema, as opposed to an increase in cardiogenic edema (1).

The decrease in cell volume observed in acute hypoxia could suggest the existence of a preapoptotic state (21), and in fact, initiation of apoptosis correlates with cleavage and disassembly of intracellular and extracellular components of adherent junctions (11). However, in our preparation, we only found a small increase in caspase-3 (Western-blotting) a mild preapoptotic sign, although the corresponding immunohistochemistry reaction was negative.

We found an increase in the production of plasmalogen that act as endogenous antioxidant (34) and could buffer the decrease in glutathione induced by hypoxia (13). We found no increase in lysophospholipids (1), suggesting no activation of PLA_2. Furthermore, lipid peroxidation was not found to be significantly increased, as assessed by a colorimetric assay of malondialdehyde (1).

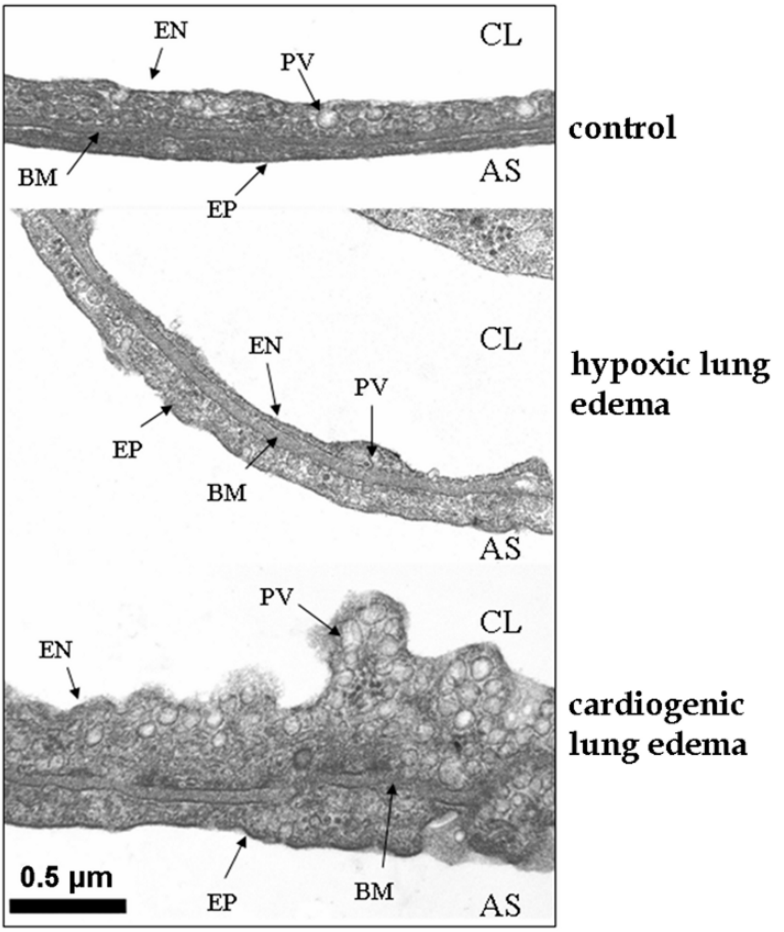

Figure 11. Morphology of the air-blood barrier at high magnification to show changes induced by hypoxic or cardiogenic lung edema. EN: endothelium, BM: basement membrane; EP: epithelium; CL: capillary lumen; AS: alveolar space; PV: plasmalemmal vesicles

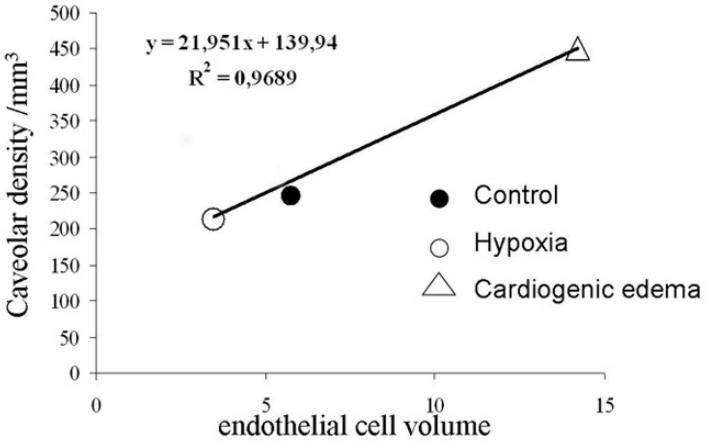

Figure 12. Regression between number of plasmalemmal vesicles per unit volume of endothelial cells plotted vs the volume of endothelial cells in control (closed circle) and in hypoxic (open circle) or cardiogenic (open triangle) interstitial edema.

CONCLUSION

Interstitial edema represents a condition whereby a negligible increase in extravascular water is maintained due to a major increase in parenchymal stresses, this in turn reflects the high elastance of the lung tissue due to the macromolecolar organization of large matrix proteoglycans (CS-PG). Hypoxia causes fragmentation of CS-PGs of the extracellular matrix and of HS-PGs of the basement membrane: the result is a decrease in tissue elastance and an increase in permeability of the endothelial and epithelial barriers. When the overall PGs fragmentation overcomes a critical threshold, severe lung edema rapidly develops, as unopposed filtration largely exceeds lymphatic drainage. We provide the evidence for a prompt lung cellular response to stimuli arising at interstitial level when microvascular filtration is increased. Indeed, endothelial and epithelial cells are in a highly deformed state because of their attachments to the extracellular matrix and to the neighboring cells, accordingly, the tensional behavior of a "hard-wired" cytoskeleton (12) might put these cells in a good position to respond promptly to mechanotransduction. The rigidity of the interstitial tissue (18) adds efficiency to the cellular response. We interpret these responses as a fine mechanism to detect minor increases in extravascular water and to promote the reparative process (1, 2, 4). Recovery from severe lung edema requires indeed that the extracellular integrity is restored. In case of hypoxic lung edema, the cellular responses can be regarded as a specific "hypoxia sensing" function mediated by mechanical stimuli arising from the extracellular matrix.

ACKNOWLEDGEMENT

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Chapter 12

HYPOXIC INHIBITION OF ALVEOLAR FLUID REABSORPTION

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Abstract: Alveolar hypoxia occurs during ascent to high altitude and is also observed in patients with ARDS and acute hypoxemic respiratory failure, in which alveolar flooding is associated with a decrease in edema fluid clearance and increased mortality. The mechanisms that lead to the impairment of alveolar fluid clearance are not completely understood. Alveolar fluid reabsorption is accomplished mostly by active Na^+ transport across the alveolar epithelium which creates an osmotic gradient responsible for the clearance of lung edema from the alveolar spaces. *In vivo* and *in vitro* hypoxia inhibits both the epithelial sodium channels, responsible for the apical sodium entry, and the basolateral Na,K-ATPase, responsible for Na^+ extrusion. We have shown that acute hypoxia inhibits Na,K-ATPase function by promoting its endocytosis from the plasma membrane to intracellular compartments. This process is mediated by the generation of mitochondrial reactive oxygen species (ROS) as shown by pharmacological and genetic approaches. Hypoxia and ROS promote the PKC-zeta dependent phosphorylation of the Na,K-ATPase alpha subunit triggering its endocytosis in a clathrin–AP2 dependent process. The phosphorylation occurs at the Ser-18 in the alpha subunit N-terminus, and mutation of this serine prevents both the decrease in function and the endocytosis. More prolonged hypoxia causes the ubiquitination and degradation of Na,K-ATPase. Thus, methods that counterbalance the inhibition of edema clearance during hypoxia and improve the lung's ability to clear pulmonary edema are needed. As such, a better understanding of the mechanisms that increase Na,K-ATPase function, (i.e., activation of dopaminergic or adrenergic receptors, gene transfer) may lead to the development of therapeutic approaches to upregulate the Na-K-ATPase function and increase edema clearance.

Key Words: alveolar epithelia, Na transport, Na,K-ATPase

ALVEOLAR FLUID REABSORPTION

Oxygen is exchanged across the alveolo-capillary barrier, and at sea level with normal ventilation the alveolar O_2 pressure is ~100 mmHg. Even though this is the highest pO_2 detected in the body, during many pathophysiological conditions alveolar hypoxia may develop. For example, during ascent to high altitude, a decrease in alveolar oxygen tension occurs as a consequence of decreased barometric pressure which can lead

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to high altitude pulmonary edema (HAPE), characterized by flooding of the alveolar space (24). Alveolar hypoxia can also develop as a consequence of hypoventilation, (caused for example by airway obstruction) or pulmonary edema from acute respiratory distress syndrome (ARDS), acute lung injury (ALI) or congestive heart failure (38, 39). Alveolar flooding leads to an increase in alveolar lining fluid volume which causes a life-threatening impairment of gas exchange (37). The reabsorption of edema fluid from the alveolar space is necessary for the resolution of ARDS/ALI and for the patient to survive (39). While the mechanisms of pulmonary edema formation have been well established, the mechanisms that impair alveolar fluid clearance are comparatively less understood (16).

The alveolar epithelium is composed of two cell types: the squamous alveolar type I (ATI) and the cuboidal type II (ATII) cells. These cells form a tight barrier which is relatively impermeable and keeps the alveoli "dry" so optimal gas exchange occurs (20). Both types of cells participate in the active reabsorption of alveolar fluid (29). The primary mechanism driving alveolar fluid reabsorption (AFR) is active Na^+ transport across the alveolar epithelium, which produces an osmotic gradient responsible for the clearance of lung edema from the alveolar spaces (18, 19, 26). As depicted in Figure 1, **s**odium uptake occurs on the apical surface of alveolar epithelial cells (AEC), mostly through amiloride sensitive epithelial sodium channels (ENaC) channels (5, 26).

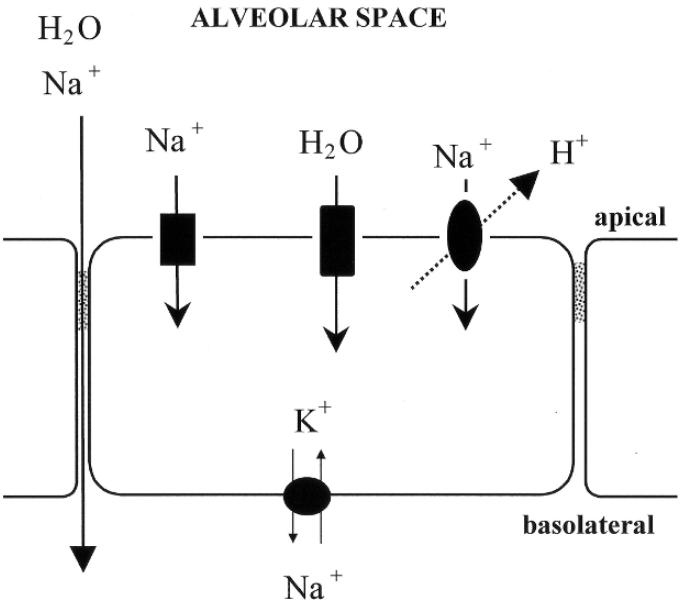

Figure 1. Mechanism for Vectorial Na+ transport.

$$ATP + 3Na^+ \text{ in} + 2K^+ \text{ out} \rightarrow ADP + Pi + 3Na^+ \text{ out} + 2K^+ \text{ in}$$

Subsequently, Na^+ is actively extruded from the basolateral surface into the lung interstitium by the ouabain sensitive Na,K-ATPase (2, 14, 31). This active vectorial sodium movement generates an osmotic gradient, which leads to the movement of

water from the airspace into the interstitium. The ENaC is a heteromultimeric protein usually formed from three homologous subunits: α, β and γ (25). The Na,K-ATPase is an heterodimeric transmembrane protein composed of an α and β subunit which is responsible for maintaining the Na^+ and K^+ gradient across the plasma membrane by pumping sodium out the cell in exchange for potassium at the expense of ATP hydrolysis (35). The catalytic alpha subunit has the binding sites for Na^+, K^+, ATP and the specific inhibitor ouabain (35). Out of the four alpha subunits identified, only the alpha 1 and alpha 2 are expressed in the alveolar epithelium (3, 22, 30). The β-subunit is a glycosylated protein and is responsible for the membrane insertion and the activity of the heterodimer (27).

HYPOXIA AND ALVEOLAR FLUID REABSORPTION

Several studies indicate that hypoxia *in vivo* and *in vitro* alters Na^+ transport by down-regulating the activities of both the Na^+ channels and the Na,K-ATPase (11). The mechanisms regulating the hypoxia-induced inhibition of Na^+ transport protein activity depends on the duration and the severity of the hypoxic exposure. Rats exposed to 8% oxygen for 24h had significantly reduced rates of alveolar fluid reabsorption as compared with their normoxic controls (23). In these experiments the Na,K-ATPase activity and α_1 subunit expression at the basolateral membrane were reduced as well as the total content (plasma membrane + intracellular compartments). *Ex vivo* experiments showed that by reducing the pO_2 from 100 to 60 or even 40 mmHg for 1h, AFR and Na,K-ATPase-α_1 subunit activity and abundance were also reduced (23).

Transepithelial Na^+ transport is inhibited in both A549 human epithelial adenocarcinoma cells and in primary rat epithelial ATII cells upon exposure to hypoxia (11), and experimental evidence suggests that long-term exposure to severe hypoxia down-regulates ENaC and Na,K-ATPase at both the mRNA and protein levels (28, 40). The decrease in the mRNA and protein expression of the ENaC subunits occurs after 3 h of exposure with a maximum decrease after 12 h (11). For longer exposures, the decrease in amiloride-sensitive Na^+ uptake was associated with a reduction of ENaC α-subunit protein synthesis (11).

We have demonstrated that short-term exposure of alveolar epithelial cells to severe hypoxia induced a time-dependent (as early as 15 min) decrease in the number of Na,K-ATPase molecules at the plasma membrane (15). This decrease in Na,K-ATPase abundance at the plasma membrane was not due to the degradation of the Na,K-ATPase, since there was no change in the total cell Na,K-ATPase protein abundance, suggesting that the Na,K-ATPase molecules were endocytosed (15).

ROLE OF REACTIVE OXYGEN SPECIES

The most important role for oxygen is the synthesis of ATP via the mitochondrial electron transport chain. Exposure of cells to moderate levels of hypoxia increases the production of mitochondrial Reactive Oxygen Species (ROS) at the semiubiquinone site of the electron transport chain, where an electron is transferred to O_2 to produce superoxide ($O_2^{\cdot -}$, Figure 2)(8, 17, 21). Superoxide generated within the mitochondrial matrix is converted to H_2O_2 by superoxide dismutase (SOD), and H_2O_2 can then be degraded by glutathione peroxidase (Figure 2). We and others have observed oxidation of the oxidant-sensitive fluorescent dye 2′,7′-dichlorofluorescein diacetate in cells and tissues during hypoxia (7, 15). In alveolar epithelial cells, the increase in reactive oxygen species production during hypoxia can be prevented by pharmacological inhibitors of the complex I and II of the mitochondria electron transport chain and by re-oxygenation, but not by inhibitors of complex III (15). Moreover, it has been proposed that during hypoxia, ROS are produced at the Q-cycle during the electron transport (6). Treatment of alveolar epithelial cells with antioxidants such as N-acetyl cysteine or catalase prevented not only the increase in ROS production but also blocked the hypoxia-induced decrease in Na,K-ATPase activity and protein abundance (15). In this context, the exogenous addition of H_2O_2 mimicked the effects of hypoxia on Na,K-ATPase activity and protein abundance (15). Furthermore, in ρ^0-A549 cells, a cell line incapable of mitochondrial respiration, and thus unable to generate ROS under hypoxic conditions (7), the effects of hypoxia on Na,K-ATPase were prevented (15). These effects were reproduced in an animal model, where in rats overexpressing the superoxide dismutase the effects of hypoxia on alveolar fluid reabsorption and Na,K-ATPase where lost (23).

Exposure to hypoxia for longer periods of time results in the degradation of both the plasma membrane and total cellular pool of Na,K-ATPase (12). The plasma membrane (active-ATP consuming) Na,K-ATPase molecules are degraded much faster (half life 2h) than the intracellularly stored (inactive) Na^+-pumps (half life longer than 24h). The hypoxia-induced plasma membrane Na,K-ATPase degradation is also mediated by an increase in the generation of mitochondrial ROS (12). Infection of alveolar type II cells with adenoviruses coding for different mitochondrial ROS scavengers (SOD, glutathione peroxidase or siRNA against the Rieske Fe-S protein) or the use of ρ0-cells prevented the hypoxia-induced degradation of the plasma membrane Na,K-ATPase (12).

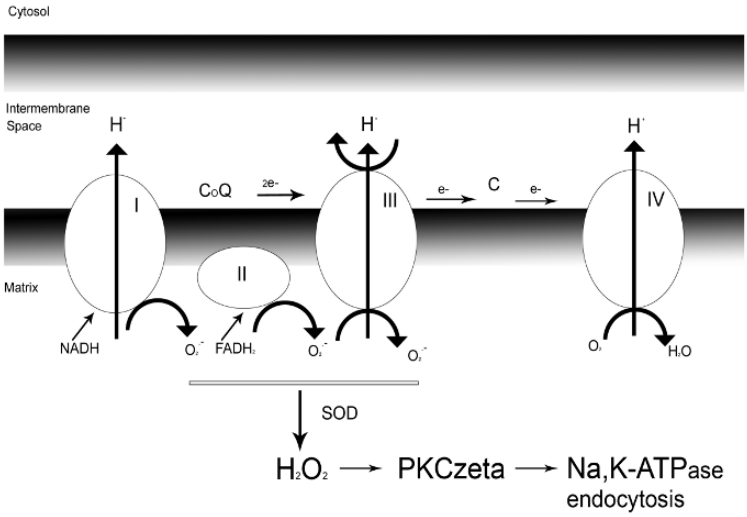

Figure 2. Mitochondrial ROS generation during hypoxia and its role in Na,K-ATPase endocytosis. See text for details.

ROLE OF PROTEIN KINASE C

It has been proposed that the ROS generated during hypoxia act as signaling messengers activating diverse cellular responses including the stabilization of hypoxia inducible factor 1 (HIF-1α) (8). In this sense, the treatment of ρ0-A549 cells with H_2O_2 produces the endocytosis of Na,K-ATPase suggesting that in alveolar epithelial cells the ROS generated during hypoxia act as messengers downstream the mitochondria activating signaling pathways. It has been previously reported that phosphorylation by protein kinase C at the Ser-18 residue of the Na,K-ATPase α_1-subunit triggers the endocytosis of the Na,K-ATPase in response to G protein-coupled receptor stimulation in renal epithelial cells (10). Treatment of alveolar epithelial cells with PKC-ζ inhibitors prevents the hypoxia and H_2O_2–induced Na,K-ATPase endocytosis (15). These results suggest both a role for PKC in the endocytic process and for ROS as signal transductors. Alveolar epithelial cells transfected with the Na,K-ATPase α_1 subunit mutated at the Ser-18 (S18A) do not respond to hypoxia (15) or to H_2O_2 (Figure 3A) by decreasing the Na,K-ATPase abundance at the plasma membrane. These experiments confirmed a role for PKC phosphorylation in the hypoxia induced-ROS mediated Na,K-ATPase endocytosis.

The intracellular mechanisms leading to Na,K-ATPase endocytosis during hypoxia have been partially identified. We have described that clathrin – mediated endocytosis plays a role in this process (9). Clathrin-mediated endocytosis is a process by which membrane proteins are selectively incorporated into clathrin-coated vesicles and transported to the internal compartments of the cell in response to a specific stimulus. In a simple model of this process, clathrin mediated endocytosis begins when a clathrin coat is nucleated at a site on the plasma membrane through recruitment by the adaptor protein 2 complex (AP-2). The AP-2 binds to the Na,K-ATPase α_1-subunit at a consensus motif (YLEL) located within the main cytoplasmic loop (13). Mutation of the tyr-537

in the Na,K-ATPase α_1 subunit renders the molecule unable to be endocytosed during hypoxia or after treatment with ROS (9). More recently we have reported that the actin cytoskeleton and the activation of RhoA small GTPase play a role in the Na,K-ATPase endocytosis during hypoxia.

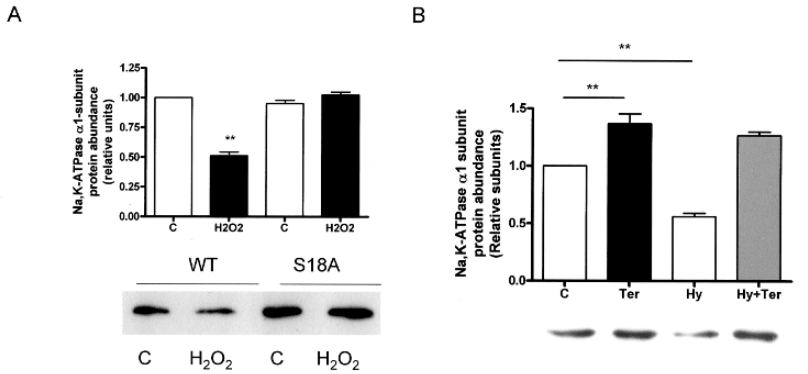

Figure 3 A: Ser18- Mutation prevents ROS-induced Na,K-ATPase endocytosis. Wild –type or S18A-α1 Na,K-ATPase –A549 cells were treated with H2O2 for 40 min. After the treatment cells were surface labeled with biotin and cell lysates (150 µg protein) were pulled down with strerptavidin beads. Na,K-ATPase protein abundance was determined by Western blot. B: β-adrenergic treatment restores Na,K-ATPase protein abundance by recruiting them from the intracellular stores. A549 cells were exposed to hypoxia for 60 min, after this exposure cells weeere trated with terbutaline for 15 min and Na,K-ATPase protein abundance was determined as in A.

β -ADRENERGIC TREATMENT RESTORES ALVEOLAR FLUID REABSORPTION DURING HYPOXIA

It has been previously reported that stimulation of the β-adrenergic receptor increases vectorial sodium transport *in vitro*, enhances the clearance of alveolar fluid and accelerates the resolution of pulmonary edema in animal models of lung injury (32-34, 36). Also, prophylactic inhalation of salmeterol decreased the incidence of high-altitude pulmonary edema in susceptible subjects by more than 50 percent (34). We have previously reported that isoproterenol increases AFR in rat lungs (32, 33) by recruiting pre-existing Na,K-ATPase molecules from internal stores to the basolateral membrane (1). We observed that rats exposed to 8% O_2 for 24 h and then treated with isoproterenol through the pulmonary circulation showed an increase in AFR which was associated with increased Na,K-ATPase protein abundance in the basolateral membrane (23). There are several mechanisms by which catecholamines may increase Na,K-ATPase activity but because of the short-term treatment, we reasoned that Na,K-ATPase stored in intracellular compartments would be recruited for insertion in the basolateral membrane. To further analyze this, we exposed alveolar epithelial cells to hypoxia and after the hypoxia exposure we treated them with the β-adrenergic agonist terbutaline for 15 min (Figure 3B). Terbutaline restores the amount of Na,K-ATPase in the basolateral membrane to the normoxic controls. In conclusion, β-adrenergic receptor agonists

improve alveolar fluid reabsorption in hypoxia-exposed lungs probably by recruiting Na,K-ATPase molecules stored in the intracellular pools to the plasma membrane.

SUMMARY

Na,K-ATPase is a highly regulated enzyme that contributes to the active Na^+ transport necessary to maintain a dry alveolar space. Alveolar hypoxia is common in patients with ARDS/ALI and decreased clearance is associated with increased mortality. During hypoxia active Na^+ transport is impaired by down regulation of both the Na,K-ATPase and the ENaC while edema accumulates due to changes in permeability of the alveolo-capillary barrier (Figure 4). Better understanding of the mechanisms that downregulate Na,K-ATPase activity during hypoxia and of the processes that counterbalance the inhibition of edema clearance and improve the ability to clear edema are needed. As such, mechanisms that improve the Na,K-ATPase function (i.e.: dopaminergic and adrenergic stimuli, gene transfer etc) represent areas of investigation toward the development of therapeutic strategies to regulate ENaC and Na,K-ATPase function and thus the reabsorption of alveolar edema.

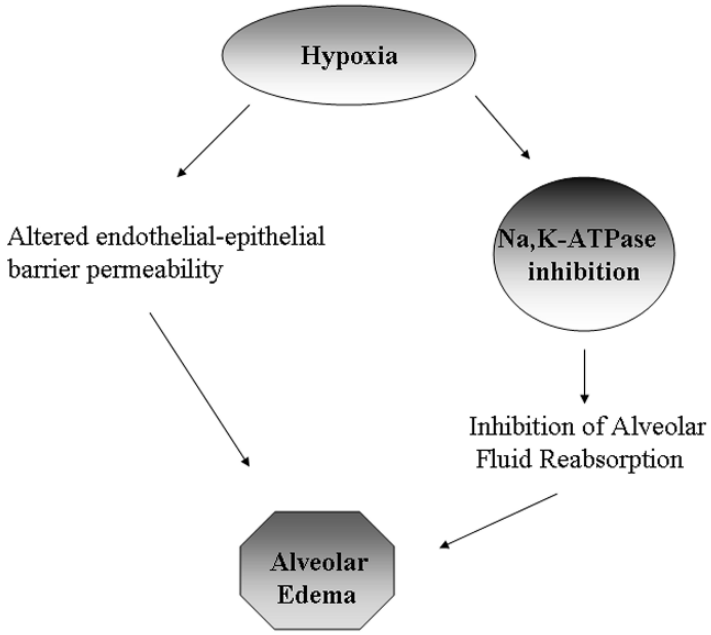

Figure 4. Mechansim for hypoxia-induced inhibition of alveolar fluid reabsorption.

ACKNOWLEDGMENTS

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Chapter 13

REGULATION AND ROLE OF NEUROGLOBIN AND CYTOGLOBIN UNDER HYPOXIA

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Abstract: Neuroglobin (Ngb) and cytoglobin (Cygb) are two novel members of the globin superfamily that are ubiquitously present in vertebrates. Their exact physiological roles are still uncertain. Here we review the expression of Ngb and Cygb, with particular emphasis on their regulation and potential role under hypoxia. Ngb expression is confined to neurons and some endocrine tissues. At the subcellular level, Ngb is associated with the presence of mitochondria and thus linked to the oxidative metabolism. Hypoxia or ischemic insults most likely do not strongly increase Ngb levels in the rodent brain. This might be explained by the fact that most mammals are not adapted to low oxygen levels. In zebrafish and turtle, however, which live in an environment with naturally changing oxygen conditions, hypoxia dramatically increases Ngb expression in the brains. We also found that hypoxia-tolerant species (e.g. the mole rat Spalax and goldfish) express more Ngb in their brains than their oxygen-deprivation sensitive relatives. These data suggest that Ngb may have a myoglobin-like role and supplies oxygen to the respiratory chain of the metabolically highly active neurons, or protect them from reactive oxygen species. Cygb is predominantly expressed in fibroblasts and related cell types, but also in distinct nerve cell populations. Cygb levels are significantly elevated at low oxygen levels in the fibroblast cell lineage. Cell culture data suggest that in fibroblasts Cygb is involved in cell proliferation, possibly in collagen synthesis. In neurons, there is evidence for an additional role of Cygb related to nitric oxide metabolism.

Key Words: globin, mitochondria, ischemia, gene regulation, reactive oxygen species

INTRODUCTION

To sustain their aerobic energy metabolism, most multicellular organisms have evolved respiratory proteins that function in the delivery and storage of molecular oxygen (O_2) (10). In man and other vertebrates, the heterotetrameric hemoglobin (Hb) in the erythrocytes of the blood transports O_2 from the respiratory surfaces (lungs, gills,

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skin) to the inner organs (Fig. 1). The monomeric myoglobin (Mb) is located mainly in cardiac and striated muscles, where it acts as a local O_2 storage device and facilitates intracellular diffusion of O_2 (31). Hb and Mb may have other, additional functions, as displayed in Fig. 1. Both Hb and Mb are members of the globin superfamily, which comprises small globular proteins with a heme prosthetic group (Fe-protoporphyrin IX) that can reversibly bind gaseous ligands like O_2, CO and NO. Globins are phylogenetically ancient proteins and have been found in all kingdoms of life.

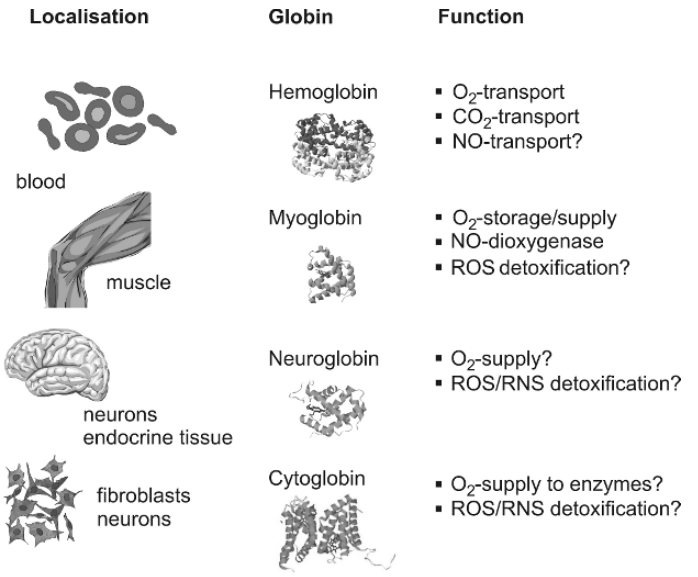

Figure 1. Distribution and putative functions of vertebrate globins.

For a long time, Hb and Mb had been considered as the only globin-types of vertebrates. However, in 2000 we identified neuroglobin (Ngb) in neuronal tissues (3) (Fig. 1). Like Mb, Ngb is a monomeric heme-protein with a molecular mass of ~16 kDa. It binds O_2 with a similar affinity of P50 (half saturation pressure) ~ 1 Torr. Ngb is preferentially expressed in the neurons of the central and peripheral nervous systems (CNS, PNS), as well as some endocrine tissues (3, 21). The highest Ngb concentration has been found in the retina, which is also the highest O_2-consuming organ of the body. More recently, cytoglobin (Cygb) was described as the fourth vertebrate globin (4) (Figure 1). Cygb is a dimer of ~21 kDa subunits with an O_2 affinity of P50 ~ 1 Torr. Its expression is confined to the fibroblast-related cell lineage (e.g., connective tissue, chondroblasts, osteoblasts, hepatic stellate cells) and some neurons of the CNS and PNS (16, 19, 24). While Cygb is a cytoplasmic protein in fibroblasts, additional Cygb is present in the nuclei of neurons (24).

It is generally accepted that intracellular globins play an important role in O_2 homeostasis of the animal cell. E.g., Mb facilitates O_2 diffusion to the mitochondria and stores O_2 for short or long term periods of hypoxia (31). It is therefore not surprising that Ngb and Cygb have been implied to be involved in various aspects of O_2-depen-

dent metabolism (for review, see Ref. 10). Besides of having a similar role like Mb in O_2 supply, Ngb and Cygb may decompose reactive oxygen or nitrogen species (ROS, RNS) or may be part of an O_2-mediated signalling chain (Fig. 1). In any case, changing O_2 partial pressures probably have significant effect on these globins. Establishing their role and regulation under hypoxia may provide evidence for Ngb and Cygb functions, and point to the biomedical significance of these proteins.

OXYGEN-DEPENDENT EXPRESSION AND REGULATION OF NEUROGLOBIN

In an aerobic organism the mitochondria consume the vast majority of the inhaled O_2, which is used by the cytochrome oxidase as an electron acceptor of the respiratory chain. Therefore, mitochondria are concentrated in regions with high metabolic activity. Due to the O_2 turnover process, mitochondria also generate various harmful ROS, particularly when the flow of O_2 is reduced under hypoxia (9). We have investigated the distribution of Ngb and mitochondria in the rodent retina (1, 23) and brain (Stephanie Mitz, Stefan Reuss, Thomas Hankeln, Thorsten Burmester, unpublished). We observed strong correlation of Ngb and mitochondria: aerobic regions with many mitochondria have much more Ngb than anaerobic regions with no or few mitochondria. These findings suggest that Ngb is actually linked to the O_2-dependent metabolism of the animal.

The connection between Ngb and oxidative cellular processes has led to the expectation that low O_2 levels should have severe effect on Ngb expression. However, there appears to be little consensus on the regulation of Ngb under hypoxia. Brains of mice and rats that had been kept for different times (from 5 h up to 2 weeks) under atmospheric O_2 levels ranging from 6 to 12% showed highly variable results (Fig. 2A). Mammen et al. (18) and Hundahl et al. (11) found no difference of Ngb mRNA levels in hypoxic and normoxic brains of two different mouse strains. Three other studies reported upregulation of Ngb (6, 8, 14). Fordel et al. (6) stated that the observed strong increase of Ngb mRNA by ~200% was not significant; most recently, the same authors observed ~70% higher Ngb mRNA levels after 48 h at 7% O_2 (8). An even stronger increase was observed in the eye (~200% after 12 h at 7% O_2). Li et al. (14) found by quantitative real time RT-PCR (qRT-PCR) and Western blotting an enhanced expression of Ngb in rat cortex at low oxygen levels (1 to 14 days sustained hypoxia [10%]). The maximum increase was ~150% on the mRNA and ~100% on the protein level. Somewhat lower induction levels were observed in rats treated with intermittent hypoxia (14). Our own experiments using Wistar rats never showed increased Ngb mRNA or protein levels under various hypoxia regimes (A. Avivi, F. Gerlach, S. Reuss, T. Burmester, E. Nevo, T. Hankeln, unpublished). It can thus be summarized that the majority of laboratories agreed that there is no strong upregulation of Ngb in rodent brains. Whether the results by Li et al. (14) and Fordel et al. (8) are due to differences in the experimental setup, data analyses or animal strains must remain uncertain.

Even when an animal is kept for an extended period under hypoxia, the actual O_2 partial pressure (PO_2) inside the brain neurons remains unknown. However, the intra-

cellular PO_2 actually controls hypoxia-regulated gene expression. In contrast, ischemic insults such as stroke reduce the blood flow and thus knowingly reduce PO_2 in total brain or in brain regions. Different experimental set-ups have been employed to study Ngb expression after ischemia/reperfusion injuries. However, neither focal (12, 29) nor global ischemia (24) induced large changes of Ngb mRNA levels in rat brains, as estimated by in situ hybridization or qRT-PCR. The observed Ngb mRNA levels in these studies ranged from 150% to 70% of the sham controls. Most recently, Shang et al. (27) reported a minor upregulation of Ngb mRNA by 45% in the brains of Mongolian gerbil at global forebrain ischemia. Our own microarray and qRT-PCR data employing rat brains after 20 min ischemia and various times of reperfusion confirm the lack of regulation of Ngb (Fabian Büttner, Christian Cordes, Frank Gerlach, Axel Heimann, Beat Alessandri, Özlem Tuereci, Thomas Hankeln, Oliver Kempski, and Thorsten Burmester, unpublished data). Thus, even when the intracellular PO_2 was reduced and other hypoxia-sensitive genes showed positive response, there appeared to be no induction of Ngb expression, at least in rodents.

By contrast, studies employing various cell culture systems are more consistent and have come to the conclusion that hypoxia < 1% O_2 leads to a significant increase of Ngb mRNA levels (6, 20, 26, 29; Fig. 2C). There are differences in the magnitudes of the observed mRNA levels, ranging from 150 to 500%, which can easily be explained by the usage of diverse experimental conditions and different neuronal cell lines. These results suggest that Ngb may in fact be hypoxia-inducible, at least in an artificial in vitro system. We evaluated the Ngb gene sequences of man, mouse and rat for putative hypoxia-responsive-elements (HREs), which potentially stimulate transcription in response to low PO_2 (32). HREs may be located in the 5' enhancer and promoter regions or downstream of the 3' UTR. HREs are usually characterized by the conserved consensus motif of the hypoxia-inducible transcription factor HIF-1 (5'-RCGTG-3'). Typically, either two HIF-1 motifs or one HIF-1 motif in combination with different co-stimulatory sequences (e.g. the EPO box; HIF ancillary sequence) are arranged within a close interval of ca. 50 bp. Several of these motifs actually occur in the mammalian Ngb genes, but none of these show a conserved HRE (32). This finding also argues against a strong hypoxia response of the Ngb gene *in vivo*, at least mediated by HIF-1. The hypoxia response of Ngb in mammalian cell culture was however reported to be dependent on the mitogen-activated protein kinase (MAPK) signal transduction pathway (33), which may interact with the HIF-pathway via the recruitment of p300/CREB transcriptional co-activator. Moreover, sequence comparisons have demonstrated the presence of a conserved hypoxia-inducible protein binding site (HIPBS) motif, which have been shown to stabilize the mRNAs of various hypoxia-responsive genes (32). Whether any of these motifs are instrumental *in vivo* remains to be established.

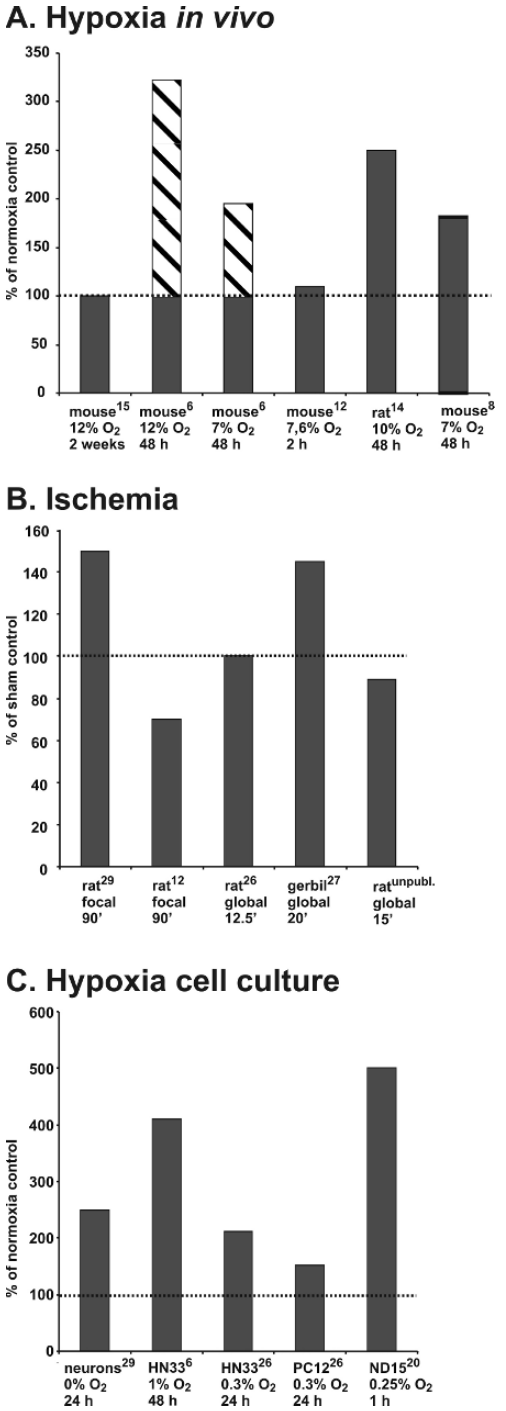

Figure 2. Hypoxia/ischemia-regulation of Ngb. Oxygen-induced changes of Ngb mRNA and protein levels were combined from the literature. In case that different induction levels were reported, the maximum number is given. Panel A shows the induction of Ngb in mouse or rat brains in vivo. The species and the hypoxia conditions (% O_2, duration) are given. Hatched bars show non-significant induction levels of Ngb (6). Panel B displays the changes of Ngb in rat or gerbil brains after ischemic insults. The species, method and duration of ischemia are given. In (A) and (B), the superscript number at the species names refers to the literature sources. Panel C shows the regulation of Ngb expression in different cell lines. Hypoxia conditions (% O_2, duration) are displayed below the panel. The superscript number at the cell lines shows the reference.

NEUROGLOBIN IN HYPOXIA-TOLERANT SPECIES

It should be considered that (at least under non-pathological conditions) most mammals will never experience a low oxygen atmosphere during their adult life. It is therefore unlikely that the brains of normal terrestrial mammals are particularly well adapted to hypoxia. By contrast, many fish and aquatic turtles live in environments with changing oxygen conditions and may actually face low O_2 levels. In fact, significant upregulation of Ngb has been observed in the zebrafish *Danio rerio* (22) and in the anoxia-tolerant turtle *Trachemys scripta* (18). In *T. scripta* brains, Ngb mRNA increased 3.5-fold after 4 h hypoxia. In zebrafish brains, Ngb mRNA and protein levels were three- to sixfold higher after 48 h at $PO_2 \sim 31$ Torr (22). No significant increase of Ngb was observed in the zebrafish eye, which is most likely due to the shutoff of metabolism in the hypoxic fish eye. Sequence evaluation shows the presence of four HREs in the D. *rerio Ngb* gene, which may be responsible for hypoxia-induced transcription. These results demonstrate that – even if there appears to be no significant hypoxia-regulation of Ngb in rodents –hypoxia-inducibility of this protein may have played a role in evolution in the adaptation of some animals to low-oxygen environments.

We further studied Ngb levels in the subterranean blind mole rat *Spalax ehrenbergi*, a mammal that can survive extended periods of hypoxia without neuronal damage. Spalax brains have constitutively higher expression levels of Ngb as compared to rats (Aaron Avivi, Frank Gerlach, Thorsten Burmester, Eviatar Nevo and Thomas Hankeln, unpublished). In the goldfish (*Carassius auratus*), which survives extended periods of hypoxia and anoxia, Ngb levels do not change upon hypoxia. However, this species has an about fivefold higher level of Ngb protein in its brain compared to the less hypoxia-tolerant zebrafish (Anja Roesner, Thomas Hankeln, Thorsten Burmester, unpublished). These observations provide additional arguments for an adaptive role of Ngb in hypoxia tolerance of these species.

IS NEUROGLOBIN NEUROPROTECTIVE?

Hypoxia causes severe damage to almost any cell. A protein that enhances O_2 supply, thus augmenting oxidative metabolism, or that depresses the hypoxia-caused injuries by other means, should therefore enhance neuronal viability under hypoxic or ischemic stress (Figure 4A). In an immortalized mouse neuronal cell line, antisense-mediated down-regulation of Ngb decreased cell viability under hypoxia, whereas additional Ngb improved cell survival (29). Administration of an Ngb antisense oligodeoxynucleotide into the mouse brain increases infarct size and worsens neurological outcome after focal ischemia (30). In turn, adeno-associated-virus-mediated Ngb over-expression improved pathology of the ischemic brain. A transgenic mouse that constitutively over-expresses Ngb showed similar results: after ischemia, the volume of cerebral infarcts was reduced by 30% (13). Interestingly, the pathological outcome of myocardial infarcts was also improved in the Ngb over-expressing mice. Ngb-mediated cellular protection also pertains to endocrine cells: Ngb protein that had been artificially introduced into isolated Langerhans' islet cells enhanced their survival (17). It is not clear, however,

whether the neuroprotective effect of Ngb is due to an O_2 supply function or some other role, like the binding of noxious reactive oxygen species (Figure 4A), (see below).

REGULATION AND INTERPRETATION OF CYTOGLOBIN EXPRESSION

In contrast to Ngb, the localization of Cygb and its cellular expression levels cannot be associated with mitochondria and general metabolic activity (25). Thus it is unlikely that Cygb is involved in O_2 supply to the respiratory chain. Nevertheless, Cygb mRNA levels were found to be significantly increased under hypoxia (Fig. 3). By exposing mice to hypoxic conditions, we have shown that Cygb is up-regulated 2 to 3 fold in heart and liver (24). Fordel et al. (6) obtained very similar results with heart, liver and muscle of mice. These results clearly demonstrate that Cygb is hypoxia-inducible in the connective tissue fibroblasts. In brain, hypoxia-induction of Cygb appears to be less pronounced (6, 14, 16): Depending on the hypoxia regime, a maximum increase of about 80% was observed in rat brains. Enhanced Cygb mRNA expression was also found in hypoxic HN33 cell lines (6); by contrast, ischemia/reperfusion regimes do not significantly increase Cygb mRNA levels in brain (Fabian Büttner, Christian Cordes, Frank Gerlach, Axel Heimann, Beat Alessandri, Özlem Tuereci, Thomas Hankeln, Oliver Kempski, and Thorsten Burmester, unpublished). Nevertheless, the general hypoxia-inducibility of Cygb is in good agreement with the presence of two conserved HREs elements in the 5' and two additional HREs in the 3' UTR of the Cygb gene region (32).

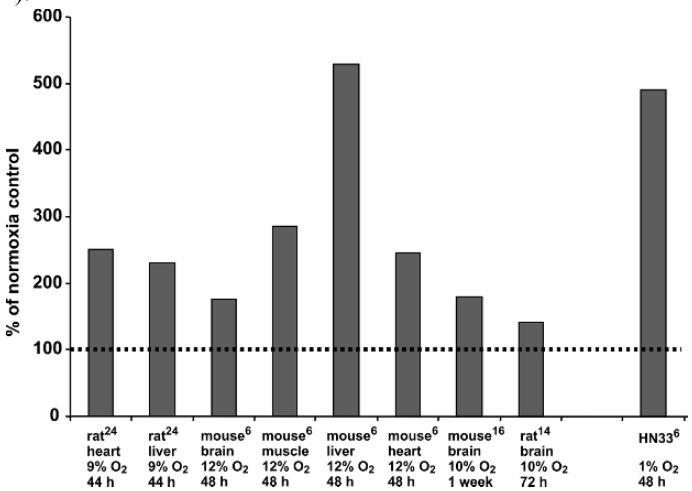

Figure 3. Hypoxia-regulation of Cygb. Hypoxia-induced changes of Cygb mRNA levels were combined from the literature. In case of different induction levels, maximum number is given. The species, specific organs (or in one case, the cell line) and the hypoxia conditions (% O_2, duration) are given. The superscript number at the species names refers to the literature source.

Expression of Cygb mRNA and protein can also be stimulated by other means: Cygb was originally discovered as protein with a strongly increased expression in activated, hepatic stellate cells during liver fibrosis (19). Cygb levels are found to be enhanced also by the activation of fibroblasts in pancreas and kidney. In primary cultures of rat hepatic stellate cells, Cygb expression is augmented by addition of recombinant transforming growth factor ß (TGFß) and platelet-derived growth factor-B (PDGF-B) and other serum factors (19). 3T3 fibroblasts transfected with Cygb show an enhanced expression of collagen and recently it was reported that Cygb has a protective effect on islet beta-cells (28).

NGB AND CYGB UNDER HYPOXIA: FUNCTIONAL AND BIOMEDICAL IMPLICATIONS

In combination with other experimental data, studies on hypoxia-regulation of Ngb and Cygb provide clues to their physiological functions (Fig 4). There is little doubt that Ngb is linked to the energy production in the mitochondria and thus O_2 consumption. In fact, Ngb may have an Mb-like role in O_2 supply to the respiratory chain, either by facilitating O_2 diffusion or by providing a short term O_2 store (Fig 4 A). Other studies have suggested other functions, which are also (at least in part) in line with the currently available data. E.g., Ngb may protect the respiratory chain from ROS or decompose ROS released specifically from the mitochondria. Lowering O_2 partial pressures impairs O_2 flow, but also leads to an increase of ROS production (9). A function of Ngb as a scavenger of ROS (or RNS) would also be consistent with the neuroprotective effect of Ngb after ischemia and reperfusion of brain tissue (13, 29, 30), when such harmful molecules are known to form. A recent study showed an enhanced survival of cell overexpressing Ngb under H_2O_2 stress (7). The same authors (8) observed in the eye a negative correlation of Ngb and H_2O_2 levels in hypoxia/reoxygenation studies, which they interpreted in terms of a ROS-scavenging function of Ngb. However, in no case we observed a timely or spatial correlation of ROS formation and known ROS-decomposing enzymes (e.g., catalase, superoxide dismustase) with Ngb expression (unpublished data). Moreover, these enzymes are readily induced by hypoxia or ischemia in rodent, but Ngb is not. We therefore consider a general enzymatic role of Ngb in ROS scavenging less likely. Brunori et al. (2) proposed that, similar to Mb in muscles, Ngb may decompose nitric oxide (NO). These authors suggest that Ngb has an NO-dioxygenase role in the context of ischemic insults, when PO_2 is low and NO levels are increased. However, the expression data rather argue for a house-keeping function of Ngb, rather than a stress-induced role. We also note that Ngb and NO-producing synthases do not significantly co-localize in brain and retina (21, 23). Therefore, a preferential NO-dioxygenase function of Ngb appears to be unlikely. Fago et al. (5) put forward the idea that the neuroprotective effect of Ngb under hypoxia is due to the reduction of ferric (Fe^{3+}) cytochrome c (Cyt c) by ferrous (Fe^{2+}) Ngb, thus preventing Cyt c induced apoptosis. This hypothesis, which based on in vitro experiments, is in line with many expression data, including the co-localization of Ngb and mitochondria, which actually release

Cyt c. Future studies must show whether an electron transfer from Ngb to Cyt c can actually occur *in vivo*. Whatever its function is, comparative physiological studies and experiments with transformed cell lines or transgenic animals demonstrate that Ngb is beneficial for neuronal survival at O_2 deprivation. Future studies will have to demonstrate whether these findings can be employed for medical applications in humans, e.g. by improving the neurological outcome after stroke.

Much less data are currently available for Cygb, but there are some clues to its function. In contrast to Ngb, Cygb is not correlated with mitochondria and thus energy production (25). A simple Mb-style function of Cygb is therefore highly unlikely. Nevertheless, hypoxia-inducibility of the gene demonstrates that Cygb is somehow linked to the O_2-dependent metabolism. This does not necessarily mean that Cygb is involved in hypoxia survival. Further evidence for Cygb function comes from the observation that – at least in fibroblasts and related cells Cygb expression is strongly correlated with collagen production (24) and that collagen expression is enhanced by Cygb (19). We therefore hypothesized that Cygb could be involved in collagen production (10, 24). E.g., Cygb may provide O_2 directly to collagen prolyl-hydroxylases (Fig. 4B).

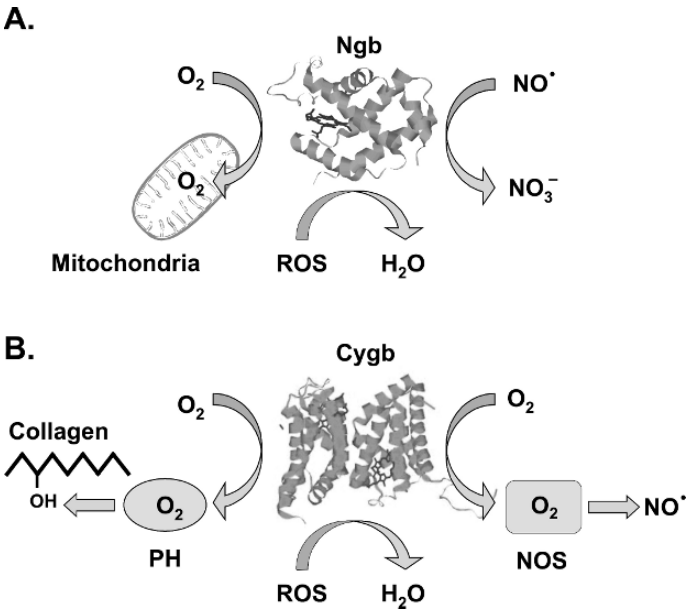

Figure 4. Putative functions of Ngb (A) and Cygb (B). Ngb may supply O_2 to mitochondria, detoxify ROS or scavenge NO. Cygb may provide O_2 to collagen prolyl-hydroxylase (PH), or to NO synthase (NOS), or scavenge ROS.

Alternatively, Cygb may protect the prolyl-hydroxylase from ROS or might participate in some unknown (O_2-dependent) signalling pathway that augments collagen synthesis. Tissue hypoxia is also a stimulatory signal in processes like osteogenesis, chondrogenesis and wound healing, in which collagens are massively deposited, there-

by possibly creating a link between the above observations on Cygb regulation. In summary, Cygb may have substantial biomedical impact due to its involvement in organ fibrosis and in the production of extracellular matrix collagens during normal tissue development and fibrotic pathogenesis. In certain neuronal cell populations that express Cygb, this protein should have another additional function. First, the subcellular localization of Cygb is different in fibroblasts and neurons, second, there is no correlation with collagen synthesis, and third, Cygb expression in brain is not (strongly) enhanced by hypoxia, in contrast to heart and liver (24). Neuronal cells that express Cygb are also positive for the expression of neuronal NO synthase (nNOS) and thus produce NO (Stefan Reuss, Sylvia Wystub, Thorsten Burmester and Thomas Hankeln, unpublished). We therefore hypothesize that Cygb in these cells either provides O_2 to nNOS for making of NO, or detoxifies NO as a dioxygenase.

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Chapter 14

MOLECULAR INSIGHTS INTO THE FUNCTIONAL ROLE OF MYOGLOBIN

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Abstract: Myoglobin is a cytoplasmic hemoprotein that is restricted to cardiomyocytes and oxidative skeletal muscle fibers. Myoglobin is a well-characterized protein and numerous studies have established that it has an essential role in facilitated oxygen transport in striated muscles. Recent strategies, using gene disruption technologies, have produced mice that lack myoglobin. These myoglobin deficient mice have a binary phenotype and a subpopulation of these mutant mice is viable and fertile. Characterization of the viable myoglobin null mice has uncovered a number of molecular and cellular adaptive mechanisms that function to promote oxygen delivery in the mutant striated muscle cell. Moreover, cellular and physiological studies, using the myoglobin deficient mouse model, support the conclusion that the functions of myoglobin include: facilitated oxygen transport, the storage of oxygen and a scavenger of nitric oxide or reactive oxygen species. Collectively, the use of genetic mouse models will further enhance our understanding of myoglobin function in normal and pathological muscle lineages.

Key Words: myoglobin, heart, skeletal muscle, knockout mouse models, oxygen transport, hypoxia

INTRODUCTION

In 1897, Mörner utilized spectrophotometry to discover the muscle pigment, which was initially termed myochrome and later renamed myoglobin in 1921 (43). Since its discovery, myoglobin has been an intensely studied cytoplasmic hemoprotein that is abundantly expressed in cardiac myocytes and oxidative myofibers in skeletal muscle. It was the first protein to have its three dimensional structure defined by John Kendrew and his colleagues using X-ray diffraction technologies (23, 24). Using these and other emerging technologies, the structure of myoglobin has been defined and continues to serve as an important model for ligand binding studies. This hemoprotein consists of a globin backbone and a prosthetic group (heme), which contains iron and allows for the reversible binding of various ligands including oxygen, nitric oxide and carbon mon-

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oxide. The ability to bind to these ligands supported the notion that myoglobin was an essential hemoprotein for cardiac and skeletal muscle function. Using gene disruption strategies, myoglobin deficient mice were generated and were capable of withstanding the cardiovascular stress of reproduction (16, 18). This molecular mouse model has been useful in the unveiling of important adaptive mechanisms that promote viability in the absence of myoglobin and supports the hypothesis that myoglobin has multiple functions within striated muscle.

In the present review, we will explore the results of studies that address the structure, tissue distribution and the functional roles for myoglobin in mammalian striated muscle lineages. We will further discuss the results of studies that have utilized transgenic and knockout technologies to modulate myoglobin expression in the mouse. These and future studies will define the regulation of important ligands such as oxygen and nitric oxide (NO) in striated muscle tissues and will serve as a platform for therapeutic applications in patients with myopathic diseases.

STRUCTURE AND FUNCTIONAL IMPLICATIONS

Myoglobin, hemoglobin and respiratory proteins (including the cytochrome proteins) are examples of hemoproteins that are capable of binding ligands such as oxygen (14, 32, 43). Since the initial characterization of the three dimensional structure of myoglobin, a number of additional studies have further enhanced our understanding of the structural organization of this hemoprotein and its role in ligand binding (1, 11, 12, 23, 24). Myoglobin is a relatively small, monomeric hemoprotein that is comprised of 153 amino acids (152 amino acids in human myoglobin). It has a backbone consisting of eight alpha helixes that wraps around a central fold that harbors the heme prosthetic group (Figure 1A). This heme group contains ferrous iron and is covalently linked with histidine amino acid residues of the protein that reside above (His-64) and below (His-93) the heme group. Xenon binding studies using X-ray diffraction have determined that the backbone of myoglobin forms four cavities (designated as Xe1, Xe2, Xe3 and Xe4). These xenon cavities are evolutionarily conserved and may function to concentrate ligands thereby modulating the kinetics of ligand binding to the heme iron (11, 12). The cavities are lined with hydrophobic, non-polar residues and have a radius greater than 5Å. Moreover, ligands may be preferentially concentrated in specific xenon pockets of myoglobin. These studies provide evidence that the structural properties of myoglobin may catalyze the binding or dissociation of specific ligands to facilitate efficient delivery of ligands in striated muscle tissues.

LIGAND BINDING

Early studies by a number of investigators (including Hill in 1936) demonstrated the capacity for myoglobin to bind oxygen. In these studies, the oxygen-binding pattern for myoglobin was hyperbolic compared to the sigmoidal shaped curve of oxygen bind-

ing to hemoglobin (Figure 1B). The hyperbolic shape of the oxygen dissociation curve for myoglobin further emphasizes the capacity for myoglobin to release oxygen in cells with low oxygen tension. These early studies supported the notion that myoglobin functions in the storage and transport of oxygen. For example, Millikan observed that myoglobin oxygen saturation was significantly decreased in the soleus muscle following interruption of the arterial blood supply compared to the resting soleus muscle (29). Moreover, the decrease in myoglobin saturation was further increased more than ten-fold following tetanic electrical stimulation of the muscle. These early studies undertaken in the 1930s supported the notion that myoglobin functions as a store for oxygen. A role for myoglobin in facilitating oxygen delivery (referred to as, "myoglobin-facilitated oxygen diffusion") from the erythrocyte to the mitochondria was firmly established in the seminal studies of Wittenberg in 1970 (41, 42).

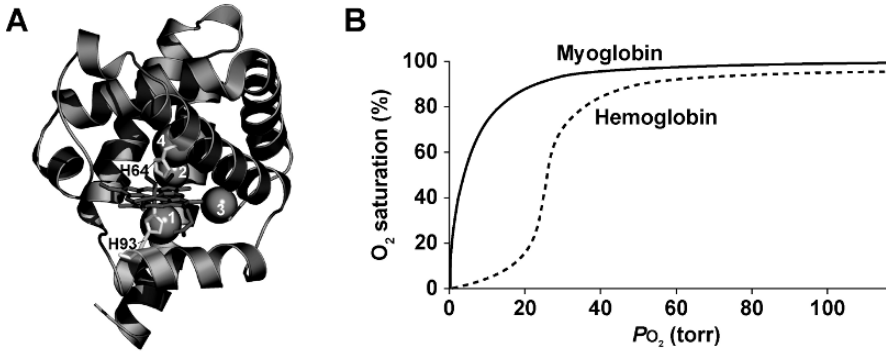

Figure 1. Structure and oxygen binding kinetics of myoglobin. (A) Myoglobin is composed of eight alpha-helices that surround a heme-binding domain. Evolutionarily conserved histidine residues stabilize the heme group (H93) or regulate the entry or exit of various ligands (H64). The prosthetic heme group is surrounded by four xenon cavities (1, 2, 3, and 4) which may serve to concentrate ligands. (B) Myoglobin and hemoglobin are both oxygen transporters; however, their oxygen-binding curves are different. Hemoglobin displays a sigmoidal-shaped oxygen binding curve while myoglobin has a hyperbolic-shaped oxygen-binding curve. (Panel A modified from *Trends Cardiovasc Med* 13: 111-116, 2003 and panel B modified from *J Exp Biol* 207: 3441-3446, 2004.)

More recently, ligand association and dissociation studies have demonstrated that myoglobin is capable of binding oxygen, nitric oxide (NO) and carbon monoxide (CO). Previous studies estimate that the oxygen tension in the heart and skeletal muscle is approximately 2.5 torr (43). At this oxygen tension, myoglobin is half-saturated with oxygen ($Mb + O_2 \leftrightarrows MbO_2$) thereby allowing oxymyoglobin to convert nitric oxide to nitrate ($MbO_2 + NO \rightarrow metMb + NO_3^-$). The ability to bind NO allows myoglobin to serve as an important regulator (i.e. scavenger) of NO bioavailability within the myocyte. Therefore, the structural organization (i.e. xenon cavities and heme binding domain) allows myoglobin to catalyze the binding of ligands and thereby regulate the bioavailability of the ligands within the myocyte even at low oxygen tension (7,8).

TISSUE DISTRIBUTION

Myoglobin is expressed early during murine embryogenesis in the cardiac and skeletal muscle lineages (Figure 2A) (13, 27). In the developing mouse embryo, the onset of cardiac expression, using in situ hybridization and RT-PCR techniques, is observed by E8.5 in the developing ventricle and by E9.5 in the myotome of the developing somite (13). By mid-gestational age, myoglobin expression continues to increase and remains restricted to the striated muscle lineages (13). In contrast to the ventricle, the onset of myoglobin expression in the atria is evident only following birth (Figure 2B) (13, 45). These in vivo embryonic and postnatal studies are complemented by the expression of myoglobin in C2C12 muscle cells. Using RT-PCR techniques, myoglobin is absent in C2C12 myoblasts, but progressively increases with differentiation and formation of the multinucleated myotubes (40). Myoglobin is also expressed in oxidative skeletal muscle myofibers (Type I fibers > Type IIa fibers > Type IIx fibers and absent in the glycolytic Type IIb fibers) in skeletal muscle (13).

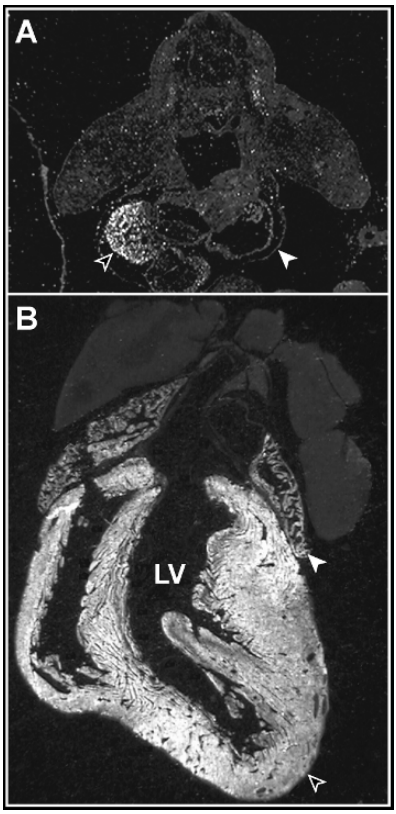

Figure 2. *In situ* hybridization of transverse sections of the embryonic (E10.0) and adult hearts probed with a ^{35}S-labeled anti-sense riboprobe for myoglobin. (A) Myoglobin expression in the embryonic ventricle is abundant but absent in the atrium. (B) There is robust expression of myoglobin in the adult ventricle and atrium. Filled arrowheads indicate the atrium while the open arrowheads identify the left ventricle (LV). (Figure modified from *Trends Cardiovasc Med* 13: 111-116, 2003

Utilizing ultrastructural immunohistochemical techniques, myoglobin was localized to the A-band and the I-band in skeletal muscle (22, 30). Importantly, mitochondria and the endoplasmic reticulum are localized to the I-band, further supporting a role for myoglobin as a reservoir or transporter of oxygen. While myoglobin is a cytoplasmic

protein, studies have demonstrated that it is excluded from both the mitochondria and the sarcoplasmic reticulum. Future studies will need to examine the localization of myoglobin under hypoxic and normoxic conditions in both cardiomyocytes and skeletal myofibers.

Myoglobin content in heart and skeletal muscle is dependent on the species, the environmental conditions (i.e. living at sea level vs. high altitude) and the level of activity. The myoglobin content was observed to be 2 mg/g wet weight in the human heart and skeletal muscle (34, 37). In contrast, mammals adapted for breath hold diving had a 30-fold increase in skeletal muscle myoglobin content (i.e. 64 mg/g wet weight in the Northern elephant seal) (31). These studies further support the notion that myoglobin is induced in response to hypoxic conditions and activity state.

General consensus supports the conclusion that myoglobin is restricted to the cardiomyocyte and oxidative skeletal myofibers. Although a recent report using cDNA microarrays and RT-PCR technologies suggested that myoglobin may have a broader expression pattern in response to hypoxic conditions (10). Fraser et al. analyzed myoglobin expression in the common carp, *Cyprinus carpio,* which is routinely exposed to "extreme environmental hypoxia" (10). Using limited techniques, the authors demonstrated that myoglobin transcripts were expressed in muscle and non-muscle lineages including the brain, liver and gill. Future studies will be necessary to determine whether this finding (i.e. expanded expression of myoglobin in non-muscle tissues) is limited to the carp or whether it is observed in other species as well.

TRANSCRIPTIONAL REGULATION OF MYOGLOBIN GENE EXPRESSION

Hemoglobin and myoglobin evolved from a common ancestral gene more than 500 million years ago. The myoglobin gene is relatively simple in structure as its organization consists of three exons and two introns. Exon 2 in the mouse and human myoglobin gene encode the heme-binding domain and have considerable sequence homology (16).

Utilizing transcriptional assays and transgenic technologies, the 2.0kb upstream fragment of the myoglobin gene has been shown to direct expression to the heart and skeletal muscle (33). The transgenic studies demonstrated that the 2.0kb promoter fragment fused to the lacZ reporter directed expression to the heart and skeletal oxidative myofibers and recapitulated endogenous myoglobin expression. Transcriptional assays using the 2.0kb upstream fragment of the myoglobin gene fused to the luciferase reporter revealed robust activity in differentiated myotubes (21). Analysis of the upstream fragment revealed evolutionary conservation of transcription factor binding motifs including a CCAC box for Sp1 binding, an A/T rich motif to bind myocyte enhancer factor-2 (MEF2), nuclear factor of activated T-cell (NFAT) response elements to bind NFAT and an E-box to bind members of the MyoD family (and other factors as well) (Figure 3) (4, 5, 21, 45). Electromobility gel shift assays, site directed mutagenesis, and transcriptional assays verified that MEF2, Sp1 and NFATs were potent

transcriptional regulators of the myoglobin gene. These and other data support the notion that MEF2, Sp1 and NFATs are responsive to calcium regulated signaling pathways and are potent transcriptional regulators of myoglobin gene expression. Further transcriptional analysis of the myoglobin gene revealed an E-box in the 5'-untranslated region of the myoglobin gene, which was important in fiber type specific transcription (4, 5, 21, 45). Mutagenesis of this E-box resulted in persistent expression in the oxidative soleus muscle but increased expression in muscles rich in glycolytic myofibers (i.e. white vastus lateralis muscle) compared to the wild-type transgene that has an intact or unperturbed E-box. These results suggest that transcriptional repressors that serve as cognate factors to bind to this E-box directly or alternatively members of the MyoD family may bind to the E-box and recruit a cofactor that represses transcription to direct myoglobin gene expression in a fiber-type specific fashion (i.e. expression in slow twitch oxidative fibers and absent expression in fast twitch Type IIb glycolytic fibers). Importantly, these studies underscore the stringent and coordinated regulation of the myoglobin gene by permissive and repressive factors to direct expression specifically to oxidative myofibers. Future studies will be necessary to further examine the transcriptional regulation of the myoglobin gene in response to additional environmental stimuli including acute and chronic hypoxia.

Figure 3. Evolutionarily conserved motifs within the 2.0kb myoglobin promoter is sufficient to regulate myoglobin expression within cardiomyocytes. The 2.0kb myoglobin promoter contains regulatory response elements [i.e. NFAT response elements (NRE), CCAC box, A/T motif, and E-box] that are recognized by transcription factors [nuclear factor of activated T-cell (NFAT), Sp1, myocyte enhancer factor-2 (MEF2)] that regulate myoglobin transcription. Mb, myoglobin.

KNOCKOUT MOUSE MODELS AND THE FUNCTIONAL ROLE OF MYOGLOBIN

To further explore the functional role of myoglobin in striated muscle we and others utilized a gene disruption strategy to engineer mice that lacked myoglobin (16, 18). The gene targeting strategy deleted exon 2 of the myoglobin gene, which encodes the heme-binding domain (Figure 4). We observed a binary phenotype associated with the myoglobin deficient embryos (28). A significant number of myoglobin null embryos were nonviable and displayed lethality by mid-gestational age. These nonviable myoglobin null embryos had a number of severe defects including a developmental delay with growth retardation, perturbed cardiac morphogenesis, vascular insufficiency (associated with diffuse hemorrhage) and cardiac failure (28). Light microscopic and ultrastructural analyses revealed failing myoglobin null embryos (E9.5 to E10.5) to have myocardial congestion, pericardial effusion and incomplete development of the

compact layer of the myocardium compared to the wild-type litter mates (28). We further observed that embryos that were haploinsufficient for myoglobin (i.e. heterozygote for myoglobin) had increased embryonic lethality (E9.5) supporting the notion that myoglobin dosage is critical for viability during this early period of cardiac morphogenesis (28).

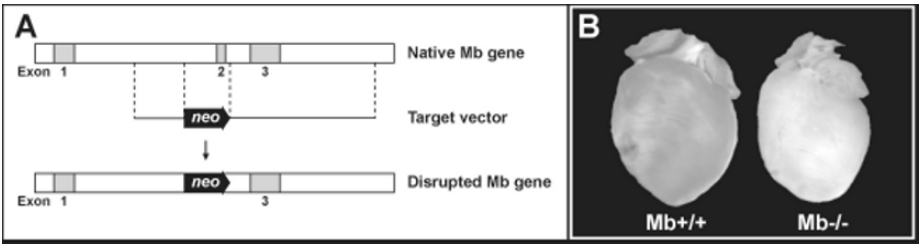

Figure 4. Generation of myoglobin deficient mice utilizing homologous recombination technology. (A) Myoglobin knockout mice were generated by replacing exon 2, the exon encoding the heme binding domain, with a neomycin cassette (neo). (B) Hearts lacking myoglobin (Mb-/-) are depigmented compared to wild-type hearts (Mb+/+) due to the absence of heme from myoglobin.

The myoglobin mutant embryos that survive this developmental period mount important adaptive mechanisms that promote viability (20, 28). The viable myoglobin mutant embryos were characterized by an induction of gene expression and increased vascularization limited to the cardiovascular system. Viable myoglobin null embryos had formation of myocardial vascularization at earlier developmental stages compared to the wild-type control (28). This increased myocardial vascularization resulted in approximately a 30% increase in capillary density in the ventricles of the E12.5 Mb null heart compared to the age matched control (28). The viable myoglobin null embryonic heart was associated with an induction of the hypoxia inducible gene program. This program included an induction of hypoxia inducible factor-1 (HIF1α), HIF2α, vascular endothelial growth factor (VEGF) and stress mediated chaperones (hsp27) (28). Presumably, this molecular program promotes cellular adaptations including increased vascularization that promote viability.

Myoglobin null mice that are viable have no evidence of premature death and are capable of surviving the cardiovascular stress of pregnancy (Figure 5). Adult myoglobin null mice have increased cardiac vascularization and an induction of the hypoxia gene program (20, 28). These molecular and cellular adaptations result in preserved oxygen consumption and cardiac performance. Specifically, the hemodynamic analyses revealed that the myoglobin mutant heart has no significant differences in cardiac output, heart rate, stroke volume or coronary flow compared to the wild-type control (28). In addition, no significant differences were observed in the mutant mice in response to either chronic adrenergic stimulation or ischemic injury (i.e. left anterior descending coronary artery ligation induced injury) compared to the respective controls (28). Collectively, these results suggest that the reprogramming of gene expression in the absence of myoglobin is necessary and sufficient to promote cellular adaptations and preserved cardiac performance.

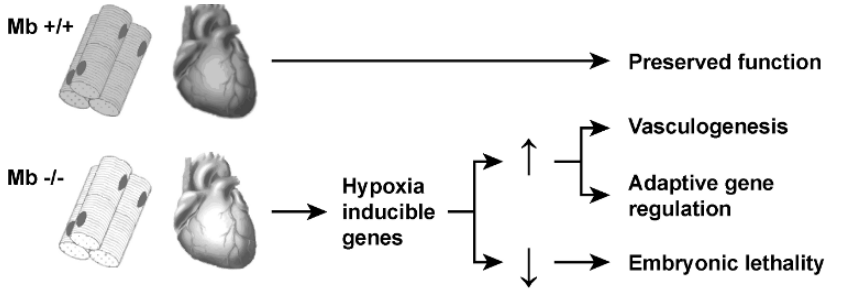

Figure 5. Cellular and molecular adaptations promote viability and preserved cardiac function in myoglobin deficient mice. A percentage of mice that lack myoglobin are viable and have preserved cardiac function due to various cellular and molecular adaptations. However, myoglobin null mice that fail to develop these adaptations die in utero between E9.5 and E10.5.

To further characterize the myoglobin deficient mouse model and unveil additional functional roles for this hemoprotein, mutant and wild-type mice were exposed to four weeks of chronic hypoxia (i.e. 10% oxygen) (25). Within seven days of chronic hypoxia, the myoglobin mutant mice had a 30% decrease in left ventricular (LV) function (compared to the wild-type control) which was persistent and was completely reversible with re-exposure to normoxic conditions. Moreover, the decreased LV function of the myoglobin deficient mouse could be completely prevented with the treatment of an inhibitor of nitric oxide (NO) synthases (25). These results support the notion that myoglobin functions, in part, as a scavenger of NO.

These data are supported by two studies from the Schrader laboratory (8, 39). Using ^{1}H nuclear magnetic resonance (NMR) spectroscopy, Flögel et al. demonstrated that with intracoronary infusion of increasing NO concentrations in wild-type murine hearts, oxygenated myoglobin (MbO_2) was increasingly reduced to metmyoglobin (metMb) with a simultaneous increase in nitrates (NO_3^-) (8, 39). However, these experiments in myoglobin null hearts did not result in the reduction of MbO_2 nor the oxidation of NO. In addition, myoglobin null hearts that were exposed to increasing concentrations of NO developed a decrease in left ventricular pressure compared to wild-type hearts exposed to similar concentrations of NO. Therefore, this study supports the hypothesis that myoglobin plays an important role in maintaining NO homeostasis. This study was further supported by Wegener et al. who observed a greater reduction in contractility in myoglobin null hearts compared to wild-type hearts upon exposure to the NO donors of the NONOate group (i.e. DEA-NONOate and DETA-NONOate) (39).

The Schrader laboratory has further demonstrated that myoglobin protects the cardiomyocyte by specifically regulating iNOS-generated NO within the myocyte (19, 44). Using ^{1}H NMR spectroscopy, Wunderlich et al. demonstrated that mice that overexpress iNOS (TgiNOS) have a significant decrease in MbO_2 signal but a concomitant increase in metMb levels indicating that there is less bioactive NO available (44). Hearts from these TgiNOS mice have preserved contractility and energetics due to myoglobin's ability to scavenge the excess NO. However, acute inhibition of myoglobin by carbon monoxide resulted in increased NO levels in TgiNOS hearts. These

hearts had depressed contractility and deranged cardiac energetics. This study was further supported by Gödecke et al. who demonstrated that mice over-expressing iNOS in the heart but lacking myoglobin (TgiNOS x Mb-/-) developed a dilated cardiomyopathy (19). Histologically, these hearts had dilated ventricles with evidence of interstitial fibrosis. The fetal gene program was activated in these hearts. Finally, the hemodynamic and metabolic profiles of these TgiNOS x Mb-/- hearts were reflective of cardiac decompensation.

Additional studies have been undertaken to examine the compensatory mechanisms of myoglobin deficient skeletal muscle (i.e. the myoglobin deficient soleus muscle) (20). Similar to the heart, myoglobin deficient soleus muscles are characterized by reprogramming of gene expression (induction of the hypoxia gene program), increased capillary density, low frequency fatigue and perturbed NO signaling (20). These studies further establish common molecular pathways and cellular adaptations in cardiac and skeletal muscle lineages in the absence of myoglobin.

Finally, there is significant biochemical data that indicate myoglobin, due to its similarity with other peroxidases, can react with peroxides and thus serve to regulate the redox state within a cell (7). However, to date, there is only one published study that supports this hypothesis in vivo. Flögel et al. recently demonstrated that isolated perfused myoglobin null hearts exposed to oxidative stress (either by the intracoronary infusion of hydroperoxide or the generation of endogenous reactive oxygen species after an ischemia-reperfusion injury) develop more hemodynamic compromise (i.e. decreased left ventricular developed pressure) and more reactive oxygen species than the control wild-type hearts (7).

Collectively, the data using the myoglobin knockout mouse model in two distinct strains support the hypothesis that myoglobin serves an essential role within the myocardium to transport oxygen, regulate NO homeostasis (in particular iNOS-derived NO) and serve as a key modulator of the redox state within the cardiomyocyte (Figure 6).

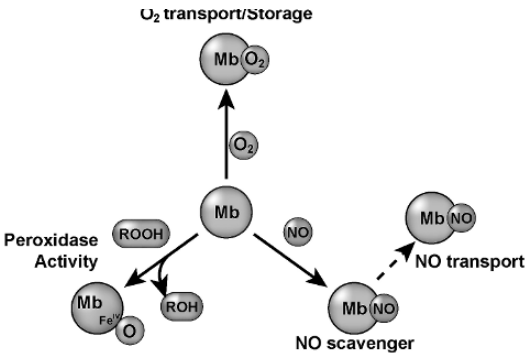

Figure 6. Functional roles of myoglobin within the myocyte. Recent studies support myoglobin's role in oxygen transport/storage and scavenging NO and reactive oxygen species. Mb, myoglobin; NO, nitric oxide.

TISSUE GLOBINS

Recent discoveries suggest that a family of tissue globins may function in a similar fashion as myoglobin in other tissues (2, 3, 6, 9, 15, 26, 27, 35, 36, 38). Neuroglobin and cytoglobin are two members of this family that share sequence homology with myoglobin and contain a heme-binding domain. Consequently, neuroglobin and cytoglobin are both capable of reversibly binding oxygen. While neuroglobin appears to be relatively restricted to the neuronal lineages, cytoglobin appears to be more broadly expressed in many adult tissues including the heart, skeletal muscle and brain. The role of these globin family members remains ill defined but the possibility exists that they may play a compensatory role, in part, in the myoglobin deficient heart and skeletal muscle.

FUTURE PERSPECTIVES

Myoglobin is a dynamic cytoplasmic monomeric hemoprotein that is tissue restricted to cardiomyocytes and oxidative skeletal myofibers. Over the past one hundred years, intense interest has focused on the functional roles of this hemoprotein. Recent studies utilizing transgenic and knockout technologies have generated mice with variable dosages of myoglobin expression. These genetic mouse models have been useful in the definition of important cellular adaptations in response to the reprogramming of gene expression that promotes viability in the absence of myoglobin. In addition, these mouse models have been useful in the definition of functional roles for myoglobin including facilitated oxygen diffusion, oxygen storage, and a scavenger of NO and reactive oxygen species. Future studies will focus on the transcriptional regulation of myoglobin gene expression in response to extreme environmental conditions (i.e. hypoxia and intense exercise). An important question to address is whether myoglobin is a direct downstream target of HIF1α. In addition, the definition of the compensatory or overlapping role(s) for tissue hemoglobins (i.e. myoglobin, cytoglobin, neuroglobin, hemoglobin, etc.) in muscle and non-muscle lineages will be explored in these genetic mutant mouse models. These studies will utilize gene disruption technology and will analyze double or triple mutant mice lacking multiple tissue hemoglobins. Ultimately, an enhanced understanding of the role of myoglobin and other tissue hemoglobins in promoting oxygen delivery and cytoprotection may be useful as therapeutic interventions in patients with congenital or acquired myopathies.

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Chapter 15

GENETIC MECHANISMS UNDERLYING REGULATION OF HEMOGLOBIN MASS

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Abstract: Hemoglobin, the sole carrier of oxygen to tissues, accounts for most cytoplasmic protein of the erythrocyte, an enucleate cell lacking protein synthesizing machinery and with limited energy metabolism. While a number of genetic mechanisms can result in decreased hemoglobin concentration in the blood, this review concentrates on those that lead to increased hemoglobin mass, i.e. polycythemia or erythrocytosis. Polycythemia may be due to a) mutations of the enzyme synthesizing 2, 3 BPG, a metabolic intermediate which regulates hemoglobin-oxygen affinity and thus oxygen delivery, b) mutation of the α or β globin genes that increase hemoglobin-oxygen affinity and thus decrease oxygen delivery, and c) mutations of the erythropoietin receptor gene or genes regulating erythropoietin production that lead to increased production of erythrocytes. *Primary polycythemias* are caused by inherited or acquired somatic mutations affecting the hematopoietic progenitors. In contrast, in *secondary polycythemia* normal progenitors are activated by external factors present in increased concentration, most commonly erythropoietin. Some hypoxia sensing disorders blur the distinction between primary and secondary polycythemias and may deserve their own category. Most polycythemias are acquired, but both primary and secondary polycythemias may be inherited. In this review we will discuss the genetic heterogeneity of individual responses to hypoxia, and the current understanding of inherited primary and secondary polycythemias.

Key Words: congenital polycythemia, disorders of hypoxia sensing

INTRODUCTION

Polycythemia is a Greek word meaning many cells in the blood. Clinically polycythemia is defined as elevated red blood cell mass (>29 ml RBC/kg in men and >27 ml RBC/kg in women). Erythrocytosis is used synonymously for polycythemia and there is no consensus yet on the use of either term. Elevation of the hemoglobin concentration can be due to either an expansion of the erythrocyte mass (absolute polycythemia) or to a reduction of the plasma volume (relative polycythemia). Absolute polycythemia can be further classified as primary polycythemia, secondary polycythemia, or polycythemia due to abnormal hypoxia sensing.

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Primary polycythemias are caused by intrinsic defects in the erythroid precursors that result in excessive response to normal stimulators of erythropoiesis. In contrast, in the secondary polycythemias cytokine responsiveness of erythroid progenitors is normal. Secondary polycythemias are driven by hormonal factors (predominantly erythropoietin but also IGF1 and cobalt) extrinsic to the erythroid progenitor cells, and the increased erythrocyte mass represents a physiologic response to tissue hypoxia or abnormal autonomous erythropoietin (Epo) production (54). Polycythemia due to abnormal hypoxia sensing blurs the distinction between primary and secondary polycythemias. Both acquired and congenital polycythemias can be primary or secondary, but polycythemia due to abnormal hypoxia sensing is usually congenital. Polycythemia vera, an acquired primary polycythemia, is characterized by clonal expansion of hematopoietic precursors, and is the most common type of primary polycythemia (63). Rarely, polycythemia vera occurs in several members of the same family wherein the phenotype is typically acquired and the inherited predisposition for its development is not fully penetrant (32). Acquired conditions that lead to increased Epo production, such as chronic hypoxia and a variety of tumors, are the most common causes of secondary polycythemias. This review will focus on progress made in understanding of congenital and inherited polycythemias including polycythemia due to abnormal hypoxia sensing and primary familial and congenital polycythemia (PFCP).

CONGENITAL POLYCYTHEMIAS CAN BE DIVIDED INTO THE FOLLOWING CATEGORIES:

1) *Congenital polycythemia* with *increased Epo levels (inherited defects in hypoxia sensing due to VHL or proline hydroxylase gene mutations)*. This group includes Chuvash polycythemia (CP), polycythemias associated with von Hippel-Lindau mutations other than the CP mutation, and polycythemia due to proline hydroxylase mutation. Only CP erythroid progenitors were well studied, and this entity has been shown to have features of both primary and secondary polycythemia. Physiologically inappropriate increased Epo levels characterize these conditions.

2) *Primary congenital polycythemia* resulting from inherited intrinsic defects in red blood cell precursors that cause increased responsiveness to Epo (primary familial and congenital polycythemia).

3) *Secondary congenital polycythemia* resulting from inherited conditions that lead to increased Epo levels. These include hemoglobin variants with high affinity for oxygen, congenitally low erythrocyte 2, 3 biphosphoglycerate levels and inherited methemoglobinemias. All these conditions are characterized by a left shift in Hb dissociation curve which in turn leads to tissue hypoxia and a physiologically appropriate increased Epo levels. Congenital cyanotic heart or lung disorders leading to tissue hypoxia and increased Epo level are also examples of secondary congenital polycythemias.

CONGENITAL POLYCYTHEMIA RESULTING FROM BOTH INCREASED EPO LEVELS (DUE TO DEFECTS IN HYPOXIA SENSING) AS WELL AS "INTRINSIC DEFECT IN RED BLOOD CELL PROGENITORS"

Congenital polycythemias resulting from defects in hypoxia sensing: Epo, the principal regulator of erythropoiesis, is produced by the renal tubular cells in response to hypoxia (1). The *EPO* gene is one of many "hypoxia-regulated" genes whose expression is controlled by the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 is composed of two subunits, HIF-1α and HIF-1β, which form a heterodimer (68); only HIF-1α is regulated by hypoxia. HIF-1 controls transcriptional regulation of multiple genes involved in diverse processes including cell proliferation and survival, glycolytic metabolism, angiogenesis, and iron metabolism. Erythropoietin, vascular endothelial growth factor (VEGF), genes encoding glycolytic enzymes, transferrin, and transferrin receptor genes are just a few of the genes that are regulated by HIF-1 (3, 60). Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway (42). The targeting and subsequent polyubiquitination of HIF1α requires von Hippel Lindau protein (pVHL), iron, O_2 and proline hydroxylase activity; this complex constitutes the oxygen sensor (23, 26). In normoxia, the prolyl residues of HIF-1α become hydroxylated, which allows pVHL to bind to HIF-1α (23). pVHL is part of a multiprotein complex that acts as a ubiquitin ligase. Subsequent to the binding of pVHL to HIF-1α, a polyubiquitin tail is added to HIF-1α. Addition of the polyubiquitin tail serves as a signal for HIF-1α to be degraded by another multiprotein complex known as the proteosome (Figure 1).

The polycythemic conditions in this group have mutations in either the *VHL* gene or a proline hydroxylase gene (49) that lead to defective hypoxia sensing resulting in inappropriately high Epo levels. Some of these disorders were shown to have an intrinsic defect in red blood cell progenitors, which leads to *in vitro* hypersensitivity (hyper responsiveness) to Epo. The molecular mechanism of this hypersensitivity to Epo remains to be elucidated. Thus disorders in this group have features of both primary and secondary polycythemia.

Chuvash polycythemia: This disorder is the first hereditary condition of augmented hypoxia-sensing to be recognized. Chuvash polycythemia (CP), the only known endemic polycythemia, is an autosomal recessive hereditary polycythemia first described by a Russian hematologist, Lydia Polyakova, in the early 1970s (52). The Chuvash people reside in the mid-Volga River region in Russia (Figure 2) and are descendents from one of the central Asian Bulgar tribes that migrated to this area approximately 1000 years ago. The Chuvash, who converted to Orthodox Christianity, remained culturally as well as geographically isolated from the surrounding Muslim tribes. This makes the Chuvash population fairly homogeneous in ethnicity. CP affects hundreds of Chuvash people, making it the most common congenital polycythemia (37). Outside of Chuvashia, CP has also been found in diverse ethnic and racial groups (46-48). Recently, Perrotta and colleagues discovered a cluster of CP on the island of Ischia (Bay

of Naples) in Southern Italy, which has a population of about fifty-five thousand. This is the first region other than Chuvashia where this congenital polycythemia is endemic (51).

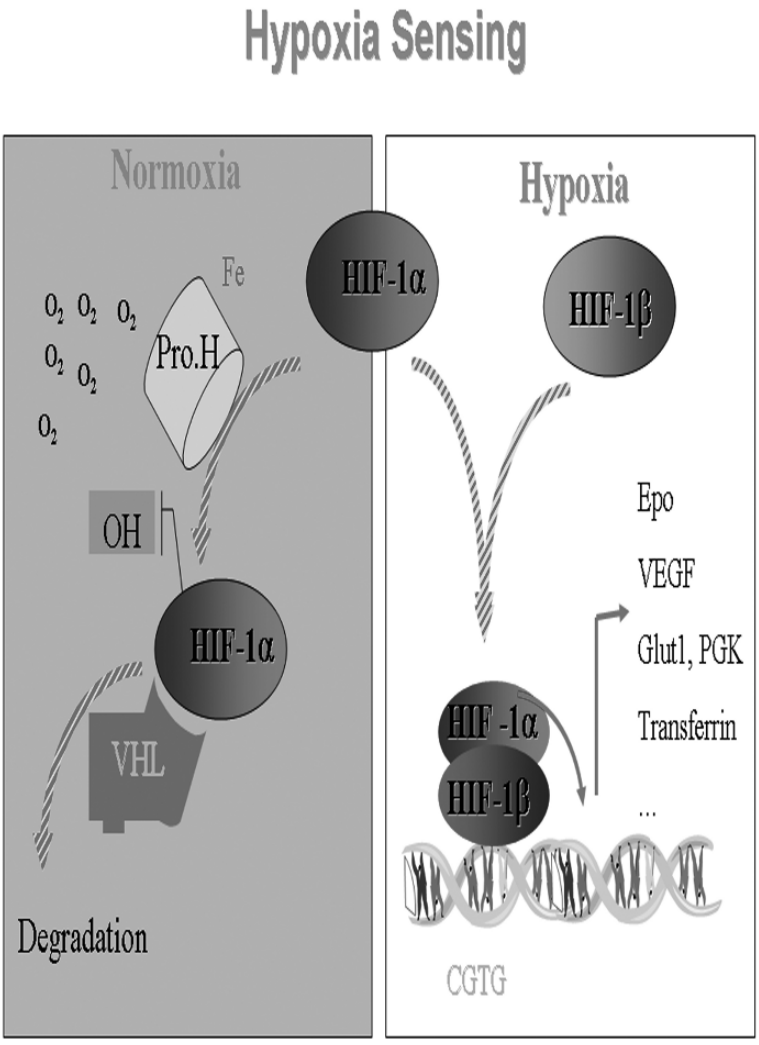

Figure 1. The protein hypoxia inducible factor 1 (HIF-1) plays a crucial role in hypoxia sensing. The cellular level of the HIF-1 α subunit is controlled by oxygen level. Oxygen activates prolyl hydroxylase (Pro H), which hydroxylates HIF-1 α. This leads to the binding of tumor supressor Von Hippel Lindau (VHL) protein, and subsequent ubiquitination of HIF-1α. In contrast, in hypoxia, HIF-1 α associates with the β subunit. The complex then binds to hypoxia responsive elements within the genome, activates associated genes, and initiates or increases production of related genes such as *EPO, VEGF,* etc.

Figure 2. The Chuvash republic or Chuvashia is located in the center of the European part of Russia with a total population of approximately 1.3 million people.

In a study of five multiplex Chuvash families with CP, a homozygous mutation of *VHL* (598C>T) was found in the affected individuals (3). This mutation impairs the interaction of pVHL with HIF-1α thus reducing the rate of ubiquitin-mediated destruction of HIF-1α. As a result, the level of the HIF-1 heterodimer increases and leads to increased expression of target genes including *EPO*, *VEGF*, *PAI* among others (2, 3). While elevated Epo levels are consistent with a secondary polycythemia, the erythroid progenitors of CP patients are also hypersensitive to Epo in *in vitro* studies (20) which is a feature of primary polycythemia.

A matched cohort study (19) of 96 patients diagnosed with CP before 1977, 65 spouses, and 79 unaffected community members of the same age, sex, and village of birth found that homozygosity for *VHL* 598C>T was associated with polycythemia,

varicose veins, lower blood pressures, elevated serum VEGF and PAI-1 (plasminogen activator inhibitor) levels, and premature mortality related to cerebral vascular events and both venous and arterial thrombotic events. In addition to polycythemia, hemoglobin-adjusted serum Epo concentrations were 10-fold higher in *VHL* 598C>T homozygotes than in controls (18, 19). Tumors typical of the classical *VHL* syndrome (see below) were not seen in CP subjects. Benign vertebral body hemangiomas (a distinct entity from hemangioblastoma) were found in significantly more patients with CP compared to controls (55% vs. 21%). Imaging studies of 33 CP patients revealed unsuspected cerebral ischemic lesions in 45% but no tumors characteristic of VHL syndrome.

As mentioned above, CP is an autosomal recessive disorder in which the patients have a germline mutation of both *VHL* alleles (2, 61). In contrast, autosomal dominant mutations of the *VHL* gene cause *VHL* syndrome (16). Heterozygotes for dominant *VHL* mutations are at increased risk of developing hemangioblastomas, renal cell carcinoma, pheochromocytoma, pancreatic endocrine tumors, and endolymphatic sac tumors when they acquire a somatic mutation in the normal *VHL* allele (39, 40). In rare cases, affected patients may present with polycythemia, a paraneoplastic manifestation of *VHL* syndrome presumably due to inappropriate production of Epo by the tumor cells; polycythemia usually resolves on removal of the tumor (16, 22).

Over 130 germline mutations in the *VHL* gene associated with classic VHL syndrome have been identified (5), but virtually all of them are 5' to the codon 200 position that is mutated in CP. Thus, CP is a unique *VHL* syndrome characterized by homozygous germline mutation of *VHL* leading to predisposition to development of thrombosis, bleeding, cerebral vascular events, and increased mortality. It is characterized by an intact response to hypoxia despite increased basal expression of a broad range of hypoxia-regulated genes in normoxia. Despite increased expression of HIF-1α and VEGF in normoxia, CP patients do not display predisposition to tumor formation. The development of hemangioblastoma and renal cell carcinoma associated with *VHL*-tumor predisposition syndrome has been proposed to be related to increased expression of HIF (9, 30) and possibly VEGF (65). Absence of predisposition to tumorigenesis in CP patients implies that deregulation of HIF-1 and VEGF may not be sufficient to cause predisposition towards tumor formation in VHL syndrome.

Homozygosity for the *VHL* 598C>T has been reported in Caucasians in the United States and Europe and in people of Southeast Asian (Indian subcontinent) ancestry (47, 48). Thrombotic complications have been reported in some of these individuals. To address the question of whether the *VHL* 598C>T substitution occurred in a single founder or resulted from recurrent mutational events, haplotype analysis was performed on subjects bearing the *VHL* 598C>T mutation and normal unrelated individuals from Chuvash, Asian, Caucasian, Hispanic and African-American ethnic groups (37). These studies indicated that in most individuals, the *VHL* 598C>T mutation arose in a single ancestor between 12,000 and 51,000 years ago. However, a Turkish polycythemic family in Turkey had a different haplotype indicating that the *VHL* 598C>T mutation in this family occurred independently (7).

Since *VHL* 598C>T homozygotes have decreased survival from thrombotic complications and thus are under negative selection pressure, the propagation of the *VHL* 598C>T mutation suggests a survival advantage for heterozygotes. Such an advantage

might be related to a subtle improvement of iron metabolism, erythropoiesis, embryonic development, energy metabolism or some other yet unknown effect (20). A protective role has been demonstrated for HIF-1α in regulating VEGF in pre-eclampsia (35, 38), a leading cause of maternal and fetal mortality worldwide (66); however, our preliminary studies suggests that this is not the case (Victor Gordeuk, unpublished observations, 2007). Another possible protective role is increased defense against bacterial infections, as the hypoxia-mediated response has been reported to be essential for the bactericidal action of neutrophils (10).

Other congenital polycythemias characterized by VHL mutation*:* Non-Chuvash germline *VHL* mutations also cause polycythemia. Some patients with congenital polycythemia have proven to be compound heterozygotes for the Chuvash mutation, *VHL* 598C>T, and other *VHL* mutations including 562C>G, 574C>T, 388C>G, and 311G>T (5, 7, 46). A Croatian boy was homozygous for *VHL* 571C>G, the first example of a homozygous *VHL* germline mutation other than *VHL* 598C>T causing polycythemia (46). Additionally, a Portuguese girl was a compound heterozygote for *VHL* 562C>G and *VHL* 253C>T (5, 20). A few cases of congenital polycythemia, known to have mutations of only one *VHL* allele, confound an obvious pathophysiological explanation. Two Ukranian children with polycythemia were heterozygotes for *VHL* 376G>T, but the father with the same mutation was not polycythemic (47). Peripheral blood erythroid progenitors from the children and father were hyper responsive to recombinant Epo in *in vitro* clonogenic assays in a way similar to what is seen in CP patients. An English patient was a heterozygote for VHL 598C>T, (48) although the inheritance of a deletion of a *VHL* allele or a null *VHL* allele in a trans position was not excluded in this patient. Subsequently, two *VHL* heterozygous patients with polycythemia were described in whom a null *VHL* allele was more rigorously excluded; (5, 7) one of these patients also had ataxia-telangiectasia (5).

Congenital polycythemias with abnormal hypoxia sensing and mutations other than VHL. A family with proline hydroxylase mutation was recently described wherein heterozygotes for this mutation had a mild polycythemia (49); however, their erythroid progenitors were not tested. Because of the small family size the possible non-erythroid phenotype could not be ascertained.

More than fifty percent of patients with congenital polycythemias with normal or elevated Epo levels do not have *VHL* mutations, and the molecular basis of polycythemia in these cases remains to be elucidated. A proline hydroxylase mutation has been excluded in some, but not in all of these patients. It is not clear why in some families, the polycythemia is dominantly inherited (41), in others recessively, and in some it is sporadic and, why in families with the same mutation the phenotype can be different (20, 21). Lesions in genes linked to oxygen-dependent gene regulation and their interacting proteins are leading candidates for mutation screening in polycythemic patients with normal or elevated Epo without *VHL* mutations and in those with *VHL* mutations with variable phenotypes.

PRIMARY CONGENITAL POLYCYTHEMIA RESULTING FROM INTRINSIC DEFECTS IN RED BLOOD CELL PRECURSORS THAT CAUSE INCREASED RESPONSIVENESS TO EPO (PRIMARY FAMILIAL AND CONGENITAL POLYCYTHEMIA)

Primary familial and congenital polycythemia (PFCP) is characterized by an autosomal dominant mode of inheritance, and less frequently, by the occurrence of sporadic cases (15, 57). This can be contrasted with Chuvash polycythemia where the inheritance is autosomal recessive. The clinical features of PFCP include the presence of isolated erythrocytosis, absence of predisposition to development of acute leukemia or other myeloproliferative disorders, absence of splenomegaly, normal white blood cell and platelet counts, low plasma Epo levels, normal hemoglobin-oxygen dissociation curve (indicated by a normal P50) and hypersensitivity of erythroid progenitors to exogenous erythropoietin *in vitro* (13, 28, 50, 55). PFCP is generally thought to be a benign condition, but it has been reported to be associated with predisposition to cardiovascular problems, such as hypertension, coronary artery disease, and cerebrovascular events, that are not clearly related to an elevated hematocrit. Association with cardiovascular disease, however, has not been described in all series (56, 58, 62) .

The distal cytoplasmic region of erythropoietin receptor (EpoR), in association with SHP-1, is required for down-regulation of Epo-mediated activation of JAK2/STAT5 proteins (11, 69), (Figure 3). Thus far, nine mutations of the Epo receptor (*EPOR*) have been convincingly linked with PFCP. All of these mutations result in truncation of the EPOR cytoplasmic carboxyl terminal leading to loss of its negative regulatory domain, resulting in a gain-of function of the *EPOR*. Three additional missense *EPOR* mutations have been described in families with PFCP, but they have not been linked to PFCP or any other disease phenotype (53). Thus, the effect of an *EPOR* mutation is not predictable. Absence of polycythemic phenotype in some patients with *EPOR* mutation is suggestive of a role played by gene modifiers or epigenetic factors in phenotypic penetrance. The mutations of the *EPOR* were found in only 12% of subjects with PFCP, suggesting that in a majority of PFCP families, mutations in genes other than *EPOR* result in defective Epo signaling and accumulation of erythrocytes (20, 27, 31).

SECONDARY CONGENITAL POLYCYTHEMIA RESULTING FROM INHERITED CONDITIONS THAT LEAD TO INCREASED EPO LEVELS

These include hemoglobin mutants with high affinity for oxygen, congenitally low erythrocyte 2, 3 biphosphoglycerate levels and congenital methemoglobinemias. All these conditions lead to a left shift in Hb dissociation curve which in turn results in tissue hypoxia leading to increased Epo levels. Congenital cyanotic heart or lung disorders leading to tissue hypoxia and increased Epo level are also examples of secondary congenital polycythemias.

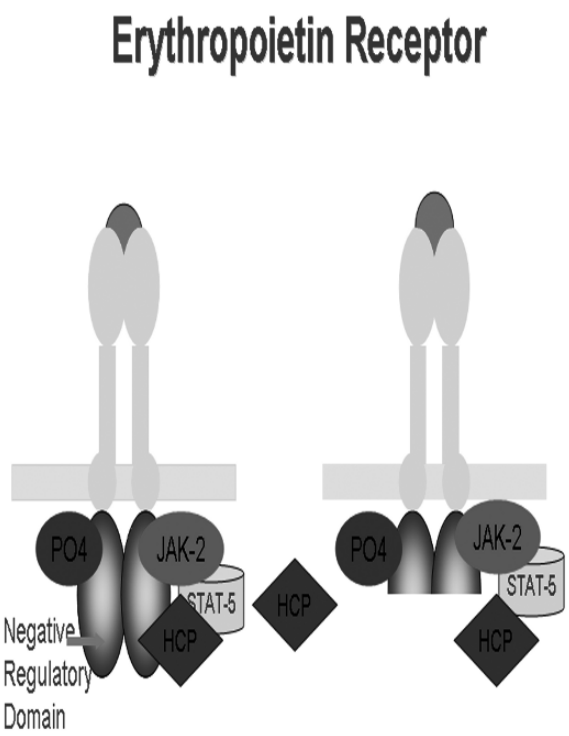

Figure 3. Erythropoietin (Epo) binding to a normal Epo receptor (EpoR) first results in interaction of a protein kinase (JAK) with the receptor which leads to phosphorylation of the receptor and initiates a cascade of signaling that results in erythroid progenitor proliferation and differentiation. This process is self regulatory however, as the activated signal transduction molecule, hematopoietic cell phosphatase (HCP) binds to the C-terminal of the EpoR which is a negative regulatory domain. This binding dephosporylates EpoR and turns off the signaling. Cells with mutated *EPOR* gene lack the portion of the EpoR that contains this negative regulatory domain. In this case Epo binds and the signal transduction pathway is activated, but since HCP can not bind to the negative regulatory domain, the receptor signaling is not turned off.

High affinity hemoglobin variants: Hemoglobin (Hb) is a tetramer consisting of two α chains and two non-α chains; the molecule also contains four heme groups. The non-α chains include the β chain of normal adult hemoglobin (α2β2), the γ chain of fetal hemoglobin (α2γ2), and the δ chain of HbA2 (α2δ2). The α globin chain contains 141 amino acids (residues) while the β globin chain contains 146 amino acids. Heme iron is linked covalently to a histidine at residue 87 of the α chain and residue 92 of the β chain (6, 44).

Oxygenation and deoxygenation of hemoglobin occur at the heme iron. The binding and release of oxygen by Hb can be graphically expressed as the Hb-oxygen dissociation curve, which has a sigmoid shape. The sigmoid shape of the Hb-oxygen dissociation curve indicates cooperative interaction between heme and oxygen. It means, as oxygenation of heme molecules proceeds, combination with further heme molecules is made easier. Oxygen affinity and Hb-oxygen dissociation is affected by blood pH, 2, 3-biphosphoglycerate (2, 3 BPG) level in the erythrocyte and temperature. Blood pH is directly proportional to oxygen affinity. Temperature and 2,3 BPG are inversely proportional to oxygen affinity (6).

Affinity of Hb with oxygen is expressed as the P50, which is the partial pressure of oxygen in blood at which 50% of the Hb is saturated with oxygen. The venous P50 can

be measured directly using a co-oximeter (not easily available) or can be calculated using a simple mathematical formula (appendix) which requires the following venous gas values: partial pressure of oxygen (venous pO2), venous pH and venous oxygen saturation (36). An abnormally low P50 reflects an increased affinity of hemoglobin for oxygen and vice versa. The P50 of a healthy person with normal Hb is 26 ± 1.3 mm Hg. The 99% confidence interval for individual observations has been reported to be 22.6 to 29.4 mm Hg. Although elevations and reductions in 2, 3-BPG level in RBC will lead to corresponding changes in P50 values, such changes are always limited to a P50 value between 20 and 35 mm Hg. A mutant Hb with high affinity should be suspected if P50 value is <20 mm Hg and presence of mutant Hb is almost certain if P50 value is <17 mm Hg (36).

Upon deoxygenation in the tissues, the hemoglobin molecule undergoes a conformational change becoming stable in a tense (T) state, which increases its affinity for oxygen. After reaching the lung vasculature, the T state hemoglobin molecule binds oxygen and undergoes a change in conformation to the relaxed (R) state. The R state has lower affinity for oxygen**.** After arriving at relatively hypoxic tissue vasculature, the R state releases oxygen following which it undergoes a change in conformation to be in T state.

The normal adult Hb (HbA) is composed of four globin chains: α1, α2, β1, β2. During oxygenation and deoxygenation, there is considerable movement along the α1/ β2 interface. Several hemoglobin mutants have substitutions affecting this interface. Other Hb variants have amino acid substitutions involving the C-terminal residues of the β chain or of the 2, 3 BPG binding sites. All these substitutions can affect the cooperative nature of oxygen binding with heme, the change from T to R state and vice versa and in turn can change the affinity of Hb for oxygen. The majority of mutations affecting oxygen affinity result in high affinity Hb variants which result in leftward shift of the dissociation curve and relative tissue hypoxia (67). There are close to 100 Hb variants, listed on the globin server, known to be associated with high affinity for oxygen (http://globin.bx.psu.edu/hbvar/menu.html accessed on March 07, 2007) (17). Compared to this there are far fewer numbers of Hb mutations known to be associated with low affinity for oxygen. All these variant Hb are inherited in an autosomal dominant manner. Hence a patient with polycythemia who has multiple polycythemic family members should raise the suspicion for a mutant Hb with high affinity for oxygen (67). A low P50 value (obtained from venous gas parameters) is supportive of high oxygen affinity Hb variant or decreased 2, 3 BPG level.

High affinity Hb variants release oxygen in the tissue relatively slowly and create relative tissue hypoxia. This leads to release of Epo from kidneys which results in increased red blood cell mass and polycythemia. At a particular level of increased red blood cell mass (which depends upon the oxygen affinity of a given variant) adequate oxygenation of the tissue is reestablished and Epo production plateaus. Hence these patients do not have progressive increase in RBC mass after achieving a certain elevated level of hematocrit. In a polycythemic patient, establishing a correct diagnosis of a high affinity Hb variant is important as these patients have normal life expectancy and do not require phlebotomy. The therapies used for polycythemia vera, should not be used in patients who have polycythemia due to mutant Hb who in our experience are frequently

misdiagnosed as such.

Congenitally low erythrocyte 2, 3 biphosphoglycerate levels: Congenitally low erythrocyte 2, 3 biphosphoglycerate (2, 3 BPG) level can occur because of deficiency of the red cell enzyme 2,3-BPG mutase (8, 34). This is an extremely rare autosomal recessive condition. This disorder should be suspected in the case of isolated polycythemia (without any features of myeloproliferative disorders such as progressive increase of RBC mass, high platelet and granulocyte count and splenomegaly), absence of a family history and low P50 (signifying high oxygen affinity). Mutant Hb still needs to be ruled out first. In these cases, the red cells will have high oxygen affinity; however, unlike high affinity Hb, the oxygen affinity of the hemolysate is normal and the level of 2, 3-BPG is very low.

Congenital methemoglobinemia: Congenital methemoglobinemia is another condition associated with high oxygen affinity of Hb and resulting increased red blood cell mass. Methemoglobin is formed when the iron of the heme group is oxidized (loses electron) i.e. converted from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. The presence of ferric heme increases the oxygen affinity of the accompanying ferrous hemes in the hemoglobin tetramer (12). This leads to a left shift in the oxygen dissociation curve, which impairs tissue delivery of oxygen. The ferric form of methemoglobin is physiologically reduced to the ferrous form via cytochrome b5 reductase (b5R). Cytochrome b5R is a housekeeping enzyme and a member of the flavoenzyme family of dehydrogenases-electron transferases. It is involved in the transfer of electrons from NADH generated by glyceraldehyde 3-phosphate reduction in the glycolytic pathway to cytochrome b5 (25, 64). FAD is a non-covalently bound prosthetic group in cytochrome b5 reductase, which acts as an acceptor as well as donor of electron. NADH donates an electron and reduces FAD to FADH (33, 45, 59). In turn, FADH reduces the heme protein of cytochrome b5, which donates an electron to reduce methemoglobin (Fe^{+++}) to hemoglobin (Fe^{++}).

There are three types of hereditary methemoglobinemias. Two are inherited as autosomal recessive traits: cytochrome b5R deficiency and cytochrome b5 deficiency. The third type is an autosomal dominant disorder, hemoglobin M (Hb M) disease in which there is a mutation of one of the globin genes. The resulting polycythemia is typically mild and its treatment is ill-advised as it would only decrease tissue oxygen delivery and lead to tissue hypoxia.

Methemoglobinemias may be clinically suspected by the presence of clinical "cyanosis" in the presence of a normal arterial PO_2 (PaO_2) as revealed by arterial blood gas analysis. Unlike deoxyhemoglobin, the dark color of the blood in methemoglobinemias does not change with the addition of oxygen. Historically pulse oximetry has been considered inaccurate in monitoring oxygen saturation in the presence of methemoglobinemia. The laboratory diagnosis of methemoglobinemia is based upon analysis of its absorption spectra, which has peak absorbance at 631 nm. A fresh specimen should always be obtained as methemoglobin level increases with storage. The standard method of assaying methemoglobin utilizes a microprocessor-controlled, fixed wavelength co-oximeter. This instrument interprets all readings in the 630 nm range as methemo-

globin; thus, false positives may occur in the presence of other pigments including sulfhemoglobin and methylene blue (29, 43). Hence, methemoglobin detected by the co-oximeter should be confirmed by the specific Evelyn-Malloy method (14). This assay involves the addition of cyanide which binds to the positively charged methemoglobin, eliminating the peak at 630 to 635 nm in direct proportion to the methemoglobin concentration. Subsequent addition of ferricyanide converts the entire specimen to cyanomethemoglobin for measurement of the total hemoglobin concentration. Methemoglobin is then expressed as a percentage of the total concentration of hemoglobin. Recently, a new eight-wavelength pulse oximeter, Masimo Rad-57 (the Rainbow-SET Rad-57 Pulse CO-Oximeter, Masimo Inc., Irvine, CA) has been reported to be accurate in measuring carboxyhemoglobin and methemoglobin. The Rad-57 uses eight wavelengths of light instead of the usual two and is thereby able to measure more than two species of human hemoglobin (4). It is approved by the US Food and Drug Administration (FDA) for the measurement of both carboxyhemoglobin and methemoglobin. In addition to the usual Spo2 value, the Rad-57 displays SpCO and SpMet, which are the pulse oximeter's estimates of carboxyhemoglobin and methemoglobin percentage levels, respectively. In a study on healthy human volunteers in whom controlled levels of methemoglobin and carboxyhemoglobin were induced, the Rad-57 measured carboxyhemoglobin with an uncertainty of ±2% within the range of 0–15%, and measured methemoglobin with an uncertainty of 0.5% within the range of 0–12%(4). However, the authors of this review have not yet had any experience with this instrument.

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APPENDIX: P50 ESTIMATION

The following mathematical formula by Lichtman and colleagues (36) allows one to estimate the P50 (formula described after the example):

An example of calculation of P50 standardized to pH= 7.4, T= 37°C from data obtained from antecubital "venous" blood. Measurement of partial pressure of oxygen (PO2), oxygen saturation (sO2) and pH in the venous blood was made at 37°C:

Example of a case with pO2=25 mm Hg; sO2=44%; sCO=2%; pH=7.37

log **p O2(7.4)** = log pO2 (observed)- [0.5 (7.40-pH (observed)]
= log 25- [0.5 (7.40- 7.37)]
= 1.3979-[0.0150]
= 1.3829

1/k = antilog (2.7 **log pO2(7.4)** × [(100 - sO2 (observed)÷sO2(observed)]
= antilog (2.7 × 1.3829)] × [(100-44÷ 44]
= antilog (3.7338) × 1.2727
= 6894.8693

Estimated P50 value = antilog [(log **1** / **k**]÷ 2.7];
= antilog [6894.8693÷2.7]
= antilog 1.4216
= 26.4 mm Hg (normal range 22.6 to 29.4).

We can calculate the P50 value for any patient if given the following venous gas values: pO2, sO2, pH; please make certain that sO2 is measured and not calculated. Please email us at Josef.prchal@hsc.utah.edu with these values.

Chapter 16

CONTROL OF THE RESPIRATORY PATTERN IN INSECTS

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Abstract: Three respiratory patterns have been described in insects: Discontinuous, Cyclic and Continuous. The Discontinuous Gas Exchange Cycle (DGC) can be distinguished by the presence of an open phase in which the spiracular valves are fully open, a closed phase during which oxygen concentrations at the tissues are lowered, thereby reducing oxidative damage, and a flutter phase in which the spiracles open intermittently for very brief periods,. The flutter phase serves to regulate internal oxygen levels at a physiologically safe level. In the Cyclic pattern, the spiracles apparently never fully close, yet a rhythmic pattern of carbon dioxide release is observed. In the Continuous pattern, no rhythmicity can be discerned and carbon dioxide release is relatively constant. In this paper I provide evidence that the three patterns described above are not distinct, but rather are a continuum. The critical parameters influencing the pattern are 1) the partial pressure of oxygen in the surrounding atmosphere, and 2) the rate of oxygen use i.e. the aerobic metabolic rate. Insects exhibit the DGC when the tracheal system is capable of delivering oxygen during the open phase at a rate faster than it is consumed by oxidative metabolism. The minute to minute balance between these two processes, one delivering oxygen the other removing it, determines the pattern of respiration exhibited by the insect at any given time.

Key Words: insect respiratory patterns, discontinuous, cyclic, continuous, oxygen, metabolic rate

INTRODUCTION

The respiratory system of insects consists of tracheae lined with cuticle (see for example 13). The tracheae can exchange gases with the external atmosphere through openings termed spiracles. Internal to the spiracles are large tracheal trunks, often associated with distensible air sacs. These sacs serve as air reservoirs within the insect's body and, if compressed, can serve to move air forcibly about the system. Tracheae branch off of the tracheal trunks in a dendritic fashion dividing repeatedly into finer and finer tubes. The tiny, terminal tips of these branches are termed tracheoles. These lie

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adjacent to the cells and are the principal site of gas exchange with the metabolically active tissues. The tracheae are filled with air and the transport of gases along their length occurs both by diffusion and by pressure pulses generated by somatic muscles in the insect's body (11, 13, 14).

The exchange of gases between the atmosphere and the insect's internal organs is not continuous. The spiracular openings on the external surface of the insect are equipped with valves which are capable of sealing off the tracheal spaces from the atmosphere. In addition, insects can generate both sub- and super-atmospheric pressure in their body fluids through the action of heart muscle and skeletal muscles in the three body segments and limbs. Because the tracheae and air sacs are compressible, pressures different from atmospheric will cause air to flow in and out of the insects through the spiracles, at least during those periods when the spiracular valves are open (14).

Comparative physiologists have long been interested in the patterns of respiration exhibited by insects (2, 9). In the recent literature, the patterns have been categorized on the basis of three basic patterns: Discontinuous, Cyclic and Continuous (3, 5,11).

THE DISCONTINUOUS GAS EXCHANGE CYCLE

The insect respiratory pattern that has received the most attention in the literature is the Discontinuous pattern usually referred to as the Discontinuous Gas Exchange Cycle (DGC) (3, 7, 11). This pattern is characterized by three distinct phases (Figure 1). In the first (the Open Phase), the spiracles are open and gases are exchanged between the intra-tracheal gases and the external atmosphere. The differences in the partial pressure of gases are such that oxygen shows net diffusion into the tracheae and carbon dioxide shows net diffusion out (7, 8, 9). In addition, during this open phase muscle contractions inside the insect can cause changes in pressure in the tracheae, leading to bulk air flow both into and out of the spiracular openings. In cockroaches, for example, these pressure changes vary tidally between positive and negative values relative to the atmosphere. This causes regular and repetitive exchanges of air into and out of the tracheae during the open phase. Presumably this tidal flow helps to speed the exchange of gases (14).

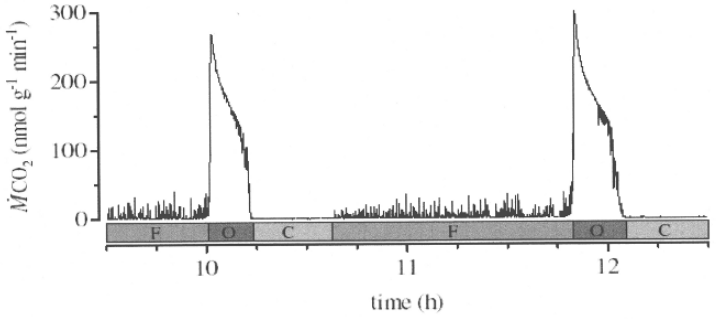

Figure 1. The phases of the Discontinuous Gas Exchange Cycle as revealed using flow-through respirometry on a pupa of the insect Attacus atlas. The phases are labeled as Closed (C), Open (O), and Flutter (F) (figure from reference 8).

Following the Open phase, many insects exhibit a Closed phase in which the spiracles are tightly sealed. During this phase, no gas exchange with the external atmosphere can occur. The Closed phase is followed by a Flutter phase. In this phase, the spiracles open repeatedly for very brief periods. In this phase, relatively little carbon dioxide and water vapor escape through the spiracles. The physiological events occurring during the short spiracular openings in the Flutter phase have been demonstrated using both flow-through respirometry, which reveals the escape of carbon dioxide in numerous short bursts, and through measurements of intra-tracheal pressure that reveal brief changes in pressure in which the negative intra-tracheal pressure approaches atmospheric pressure values. The Flutter phase is terminated again by an Open phase which initiates a new tripartite cycle as described above.

FEATURES THAT CONTROL THE THREE PHASES OF THE DISCONTINUOUS GAS EXCHANGE CYCLE

The cycle of events in the discontinuous gas exchange cycle is controlled by internal concentrations of oxygen and carbon dioxide (Figure 2). To understand this let us again begin with Open phase. During this phase, oxygen levels are increasing, due to inward diffusion from the atmosphere, and carbon dioxide levels are declining as carbon dioxide diffuses out (9). The gas-filled tracheae of insects are so effective at transferring oxygen by diffusion that the internal partial pressures of oxygen in the larger tracheae reach 18-20 kPa (7). During the following the Closed phase, there is a decline in the partial pressure of oxygen to levels around 4-5 kPa. Since carbon dioxide is more soluble in water than is oxygen, the amount of carbon dioxide that is returned to the tracheal gas space is less than the amount of oxygen removed. This leads to a net decrease in the air pressure in the tracheae during the Closed phase. Hetz and Bradley (1, 7) have argued that the purpose of the closed phase is to lower the oxygen concentration in the tracheae and thus around the tissues to reduce oxidative damage. When the partial pressure of oxygen has been lowered to this more physiologically benign level, the insects transition to the Flutter phase. During this phase the spiracles open for very short periods. The rate of "fluttering" is adjusted to maintain the partial pressure of oxygen in the 4-5 kPa range. In other words, the insect regulates the internal partial pressure of oxygen by means of quick, short openings of the spiracular valves. During this Flutter phase, the partial pressure of carbon dioxide continues to rise. There are two reasons why carbon dioxide cannot be excreted at the same rate that oxygen enters. The first is that negative air pressure in the tracheae relative to the external atmosphere cause a bulk flow of air inward each time the spiracle briefly opens. This inflow of air contains 21% oxygen. At the same time, outward diffusive movement of carbon dioxide and water vapor is slowed by the counteracting force of the inwardly moving air. The second reason is that, to the extent that oxygen and carbon dioxide can diffuse through the spiracular valves when they are open, the driving force for oxygen is greater than that for carbon dioxide. The gradient for oxygen is about 17 kPa (21 kPa outside minus 4 kPa inside) while the gradient for carbon dioxide is about 5 kPa (essentially 5 kPa

inside minus 0 kPa outside) (7, 9, 11, 12).

Carbon dioxide builds up in the insect during the Flutter phase and eventually, the carbon dioxide level reaches a critical upper limit causing the spiracles to open. This action initiates the Open phase. During this phase, the spiracles remain open long enough to remove the carbon dioxide that accumulated during the Closed and Flutter phases. This brings the insects back into respiratory homeostasis, with the drawback that oxygen has once again flooded the tracheal system. As stated above, the insects returns to a physiologically benign partial pressure of oxygen by initiating the next Closed phase (1, 7).

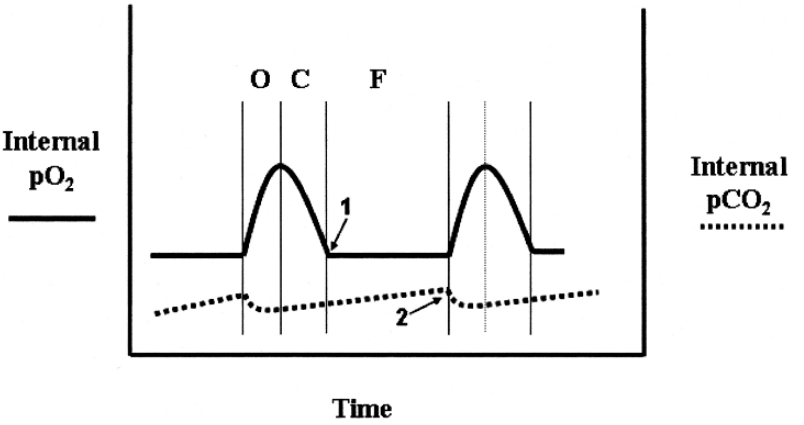

Figure 2. A representation of the changes in the partial pressures of oxygen (solid line) and carbon dioxide (dashed line) inside the insect at various times during the respiratory cycle. Note that the Flutter phase is initiated when the partial pressure of oxygen reaches a critically low level (1), while the Open phase is initiated when the partial pressure of carbon dioxide reaches a critically high level (2).

THE FACTORS THAT CONTROL THE LENGTH OF THE CLOSED PHASE

On the basis of the control system as described above, it can be seen that the length of the Closed phase is determined by the speed with which the partial pressure of oxygen can be returned to the physiologically appropriate level of 4-5 kPa. As illustrated in Figure 2, the decline in the partial pressure of oxygen is a function of two variables: 1) the amount of oxygen stored in the air and tissues in the insect at the end of the Open phase and, 2) the rate of oxygen removal during the Closed phase. The more oxygen present and/or the slower the rate of removal, the longer the Closed phase will be. This first variable, the amount of oxygen present, is determined by the anatomy of the respiratory system. If large tracheae and air sacs are present, then more oxygen will be present following the Open phase. This variable is also affected by the external partial pressure of oxygen since the internal partial pressure of oxygen cannot exceed that of

the atmosphere. The rate of removal of oxygen is determined by the rate of aerobic metabolism. During periods in which the tissues are exhibiting high rates of metabolism due to exercise or higher temperature (remember that insects are ectotherms) the oxygen will be removed more rapidly than during periods of low metabolism. The result is a pattern of CO_2 release as illustrated in Figure 3.

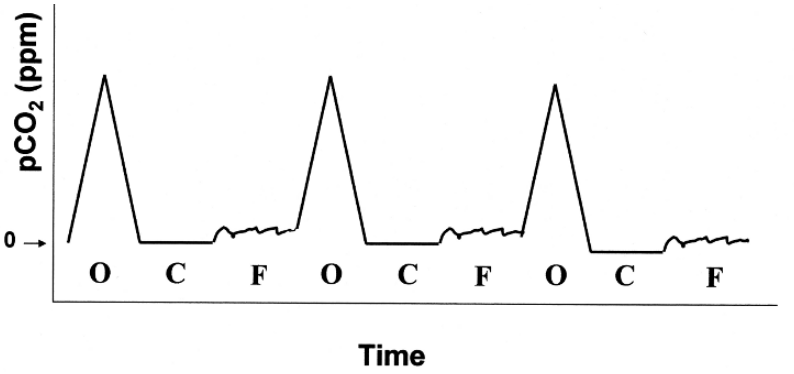

Figure 3. The Discontinuous pattern. A diagrammatic representation of the trace obtained when carbon dioxide release from the insect is measured using flow-through respirometry. The phases are labeled as Closed (C), Open (O), and Flutter (F). Note that the release of carbon dioxide goes to zero during the closed phase.

TRANSITIONS BETWEEN THE THREE RESPIRATORY PATTERNS

Most insects show transitions between the three forms of respiratory patterns depending on behavioral and environmental conditions. In a given insect at a given time, the capacities of the respiratory system and the external atmospheric partial pressure of oxygen are not variable. The most common variable affecting the respiratory pattern is therefore metabolic rate.

Let us consider what happens to an insect that is employing the Discontinuous pattern if metabolic rate is increased. The amount of oxygen contained in the insect at the end of the Open phase does not change since this is a function of the size of the tracheal system and the external partial pressure of O_2. As metabolic rate is ramped up, the Closed phase become shorter and shorter until finally it becomes so short as to not be experimentally demonstrable (Figure 4) and a cyclic respiratory pattern will be observed. In other words, the insects transition very quickly from the Closed phase to the Flutter phase in which the spiracles are opened just enough to allow oxygen to enter at the rate it is being consumed. In these insects, oxygen is being consumed faster than carbon dioxide is being released. Since carbon dioxide is continuing to build up in the insect, the critical concentration is eventually reached and this initiates the next Open phase. The cyclic pattern is characterized by Open phases in which carbon dioxide is

released in a large burst and oxygen reaches physiologically excessive levels, followed very quickly by the Flutter phase in which oxygen is allowed in at a rate equaling its rate of use in aerobic metabolism (Figure 5).

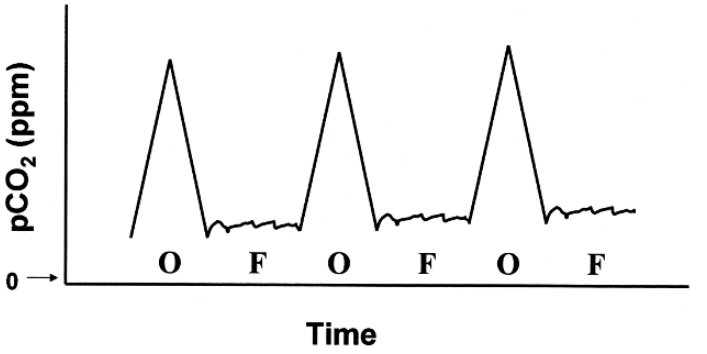

Figure 4. The cyclic pattern. A diagrammatic representation of the trace obtained when carbon dioxide release from the insect is measured using flow-through respirometry. The phases are labeled as Open (O), and Flutter (F). Note that the Closed phase is not evident and the insect transitions directly from the Open to the Flutter phase.

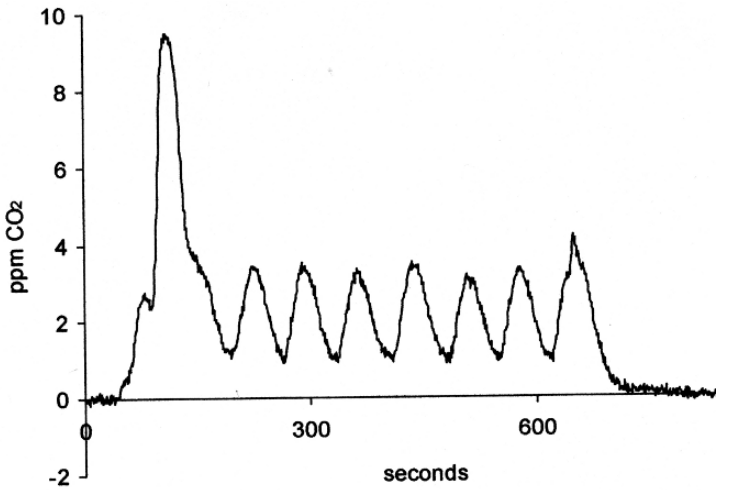

Figure 5. A cyclic pattern of carbon dioxide release obtained using flow-through respirometry on a fifth instar larva of the insect Rhodnius prolixus.

If the metabolic rate of the insects increases even more, the continuous pattern of respiration is used. In this pattern, the spiracles are opened more often and more widely as a means of providing oxygen to the tissues (Figure 6). Under these conditions, the spiracles are opened sufficiently wide to allow carbon dioxide to exit the insects at the rate it is produced. The rate of carbon dioxide release is somewhat variable but no

rhythmic pattern of release is observed (Figure 7). Instead, oxygen uptake and carbon dioxide release tend to follow metabolic rate.

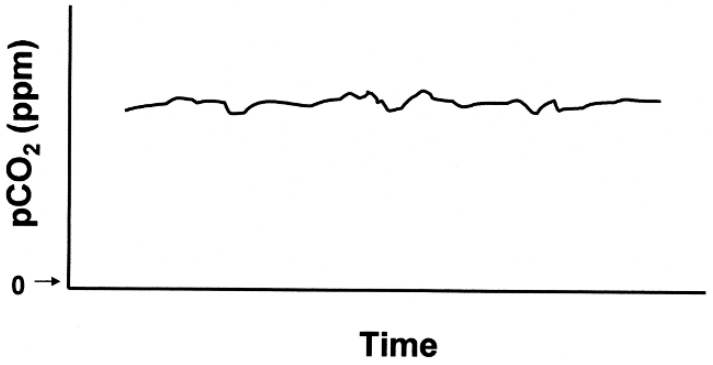

Figure 6. The continuous pattern. A diagrammatic representation of the trace obtained when carbon dioxide release from the insect is measured using flow-through respirometry. Note that the release of carbon dioxide is relatively uninterrupted and no cyclic pattern is discernable.

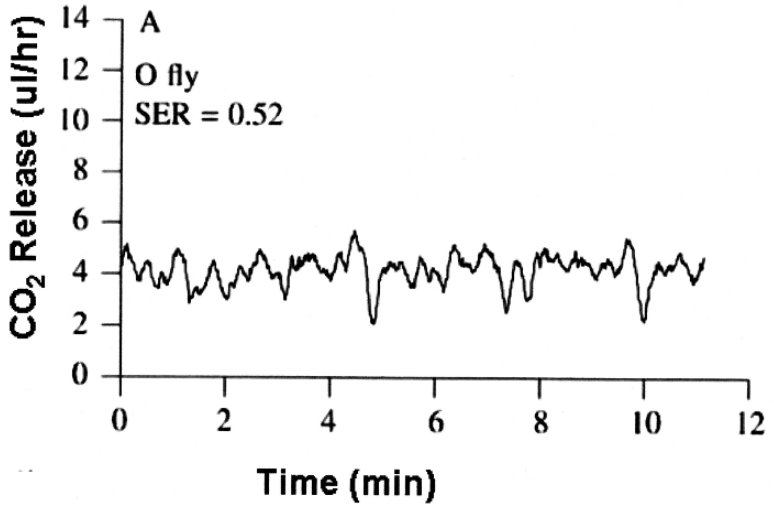

Figure 7. A continuous pattern of carbon dioxide release obtained using flow-through respirometry on an adult *Drosophila melanogaster* (figure from reference 15).

THREE SIMPLE RULES DETERMINE THE RESPIRATORY PATTERN OBSERVED IN AN INSECT

A considerable literature has arisen which describes the above patterns and which attempts to associate their occurrence to evolutionary histories, environmental factors, or the phylogenetic position of the insects is question (3, 4, 5, 6, 7, 10, 12, 14). The

explanation most generally provided is that the Discontinuous pattern, with its closed phase, serves to reduce respiratory water loss (2, 9). In this paper, I have argued that the three patterns described above are not distinct, but are in fact part of a behavioral continuum. Almost all of the variability in the respiratory patterns of insects can be explained by considering just three rules:

1. If O_2 is excessive, a closed phase will be employed to lower it.
2. Low internal levels of pO_2 (4-5 kPa) are maintained by spiracular fluttering.
3. When CO_2 accumulates to the set point, the spiracles open.

The first rule dictates that if oxygen entry during the open phase greatly exceeds the rate at which oxygen is being consumed by aerobic metabolism, a Closed phase will follow the Open phase. This Closed phase is required by the insect to lower the internal partial pressure of oxygen around the cells. The second rule indicates that the Flutter phase is used to regulate the internal partial pressure of oxygen around the cells. The length of the Flutter phase is also determined by the rate at which carbon dioxide accumulates. That rate of accumulation is a function of the metabolic rate and the rate of carbon dioxide leakage during the brief spiracular openings. Eventually, the accumulation of carbon dioxide to a critical level forces a lengthy opening of the spiracles. As metabolic rate increases, that critical level is reached more and more rapidly. As a result, changes in respiratory pattern are driven by variations in metabolic rate. Assuming no changes in the external conditions, the lowest metabolic rates in an insect will be associated with the Discontinuous pattern, slightly higher rates with the Cyclic pattern, and the highest rates with the Cyclic pattern.

I have argued in this paper that the respiratory patterns of insects are a behavioral continuum that reflects not different evolutionary histories, nor different selection pressures, but rather a simple and predictable response to variations in metabolic rate. The respiratory system of insects is extremely effective due to the capacity to distribute gases inside the insect's body through diffusion in air. As a result, at low metabolic rates the capacity to supply oxygen far exceeds the demand for oxygen and the insect must limit oxygen entry. The need to excrete carbon dioxide, however, dictates a more open spiracular condition and this tends to oversupply oxygen at low metabolic rates. It is these circumstances that dictate the Discontinuous pattern at low metabolic rates. Under unvarying external conditions, therefore, the respiratory pattern varies in a predictable pattern with metabolic rate.

What happens, however, if external conditions change? I have already alluded to the fact that conditions that affect metabolic rate (e.g. temperature, feeding, etc.) will influence respiratory patterns through their effects on the rate of oxygen consumption and carbon dioxide production. Another factor that can affect respiratory pattern is the external partial pressure of oxygen. Few environments exist in nature in which hyperoxia prevails. There are, however, many environments in which hypoxia is observed. Under these conditions, the closed phase is shortened because the amount of oxygen contained in the body following the Open phase has been reduced. We would expect, therefore to see a transition from Discontinuous to Cyclic and from Cyclic to Continuous at a lower metabolic rate than it would occur under normoxia.

Changes in the partial pressure of carbon dioxide might also influence the respiratory pattern of insects. Hypercapnia would reduce the rate of carbon dioxide leakage during the Flutter phase, accelerating the initiation of the Open phase and shortening the length of the respiratory cycle. It is well known that high levels of carbon dioxide cause the spiracles to open fully and stay open. Little work has been carried out under conditions of hypercapnia since this interferes with carbon dioxide measurements using flow-through respirometry. More work is warranted to examine the effects of elevated carbon dioxide on an insect's respiratory pattern.

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Chapter 17

EFFECTS OF INSECT BODY SIZE ON TRACHEAL STRUCTURE AND FUNCTION

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Abstract: Fossilized insect specimens from the late Paleozoic Era (approximately 250 million years ago) were significantly larger than related extant species. Geologic estimates suggest that atmospheric oxygen in the late Paleozoic Era was 35%. These findings have led to a prominent hypothesis that insect body size may be limited by oxygen delivery. Empirical evidence from developing *Schistocerca americana* grasshopper experiments suggests that larger/older animals are not more sensitive. Larger/older *S. americana* grasshoppers have a greater tidal volume at rest in hypoxia as compared to smaller animals. During jumping, larger *S. americana* grasshoppers have increased fatigue rates but the jumping muscle also consumes significantly more oxygen than smaller animals, suggesting that the tracheal system does not limit oxygen delivery. Larger/older grasshoppers were also found to have more tracheoles in their jumping muscle to promote increased diffusive oxygen delivery. Using real time x-ray synchrotron phase-contrast analysis, we have found that larger/older grasshoppers also have a greater proportional volume of abdominal tracheae and air sacs per body mass than smaller/younger grasshoppers to enhance convective oxygen delivery. To better understand if internal P_{O2} changes may be related to the increase in tracheal structure of larger/older grasshoppers, we have begun to use electron paramagnetic resonance to measure internal P_{O2} in the femoral hemolymph at rest and recovery during jumping. We have demonstrated that the femoral oxygen stores are significantly depleted during the on-set of jumping in adult *S. americana* grasshoppers. If larger *S. americana* grasshoppers have proportionally more respiratory structures throughout their body to help maintain their internal P_{O2}, the greater relative amount of body mass dedicated to respiratory structures may inhibit overall insect body size by reducing the amount of energy or space dedicated to other tissues. However, future interspecific studies are needed to better separate the effects of development and body size *per se* on the insect tracheal system.

Key Words: grasshopper, convection, diffusion, ontogeny

Hypoxia and the Circulation, edited by R.C. Roach *et al.*
Springer, New York, 2007.

INTRODUCTION

With over one million described species, insects are the most varied animal group on the planet and have adapted to almost every conceivable habitat. The evolutionary success of insects has been partially attributed to their lightweight, air-filled tracheal system. The rapid oxygen delivery of the tracheal system allows for insects to have the highest mass-specific metabolic rates in the animal kingdom (1). Oxygen delivery in the insect tracheal system occurs without the use of the circulatory system or oxygen binding molecules. Air enters the insect body through occludable spiracles, which are typically paired openings on each thoracic and abdominal body segment. Once through the spiracle, the air enters the tracheal system. Large primary tracheae branch numerous times into smaller, finer tracheal tubes. Cuticular intima in the tracheal walls provides structural support for the network. Some tracheal structures have reduced cuticular support and have expanded into large collapsible air sacs (2) that function as bellows and increase convective oxygen delivery.

The smallest tracheal tubes branch and give rise to the finest parts of the respiratory system, the tracheoles. With a diameter of about 1μm, tracheoles are the point of diffusive oxygen delivery into the tissues (3). The distal portion of the tracheole is fluid filled and oxygen diffuses through the fluid into the tissue (4). In response to increased activity and greater oxygen demand, it is believed the tracheolar fluid is withdrawn into the tissue to promote more rapid oxygen delivery (4); however, the specific mechanism is still unknown. Oxygen delivery is also maximized by matching the tracheolar density with the oxygen needs of the tissue. For example, insect flight muscles have a greater number of tracheoles than less aerobic tissues (3).

Prominent hypotheses have suggested that insect body size maybe limited by oxygen delivery problems associated with the tracheal system (5). Fossil evidence from the late Paleozoic (~ 250 million years ago) suggests that insects were significantly larger than extant relatives. For example, Paleozoic dragonflies were believed to have a wingspan of 70 cm, nearly seven-times larger than the wingspan of the largest known extant dragonfly (6). The increased body size was also seen in nearly every insect class, millipedes, spiders, and amphibians. Graham et al. (5) have hypothesized that the increased body size was due to increased atmospheric oxygen levels (35%) during the late Paleozoic, which would have provided a greater gradient for diffusive oxygen delivery through the tracheal tubes. This hypothesis is based on a diffusive model of insect oxygen delivery and does not account for convective gas exchange within the tracheal tubes.

EFFECTS OF BODY SIZE AND ONTOGENY ON OXYGEN SENSITIVITY AND PERFORMANCE

Since it is not possible to travel back to the Paleozoic, a corollary of the hypothesis that insect ‘gigantism’ was facilitated by increased atmospheric oxygen is to compare the sensitivity, performance and tracheal design of extant insects of differing body sizes

to determine if larger insects have more problems with oxygen delivery. Most studies have utilized body size changes during ontogeny (7-11). The most prominent model system for studying insect ontogeny and respiratory physiology is the grasshopper (2) because it undergoes hemimetabolous metamorphosis (juveniles and adults have similar body plans), shows a two hundred-fold increase in body size over just seven weeks (9), and the respiratory system has been extensively studied since August Krogh's early work (13).

Many lines of evidence suggest that in contrast to the prediction based on the 'gigantic insect' hypothesis, larger/older insects have greater oxygen delivery capacity than smaller/younger animals. Resting adult *Schistocerca americana* grasshoppers have greater safety margins for oxygen delivery in hypoxia (7). During activity, larger/older insects do not appear more limited by oxygen availability. Feeding and metabolic rates were not sensitive to atmospheric oxygen in *Manducta sexta* caterpillars, regardless of age or size (12). During repeated jumping, while older grasshoppers did fatigue more rapidly (8), the oxygen consumption and power produced in the jumping muscle was significantly greater in larger/older grasshoppers (9). Furthermore, when jumped at various atmospheric oxygen levels, larger/older grasshoppers were not oxygen limited (9). Future studies comparing the effects of interspecific changes in body size on oxygen sensitivity and performance are needed to separate out developmental and body size effects *per se* on oxygen delivery in insects.

EFFECTS OF ONTOGENY AND BODY SIZE ON TRACHEAL DESIGN AND FUNCTION

Developmental changes in insect body size have been shown to affect tracheal structure and function. In the grasshopper, *S. americana*, developmental changes in the size of transverse abdominal tracheae suggest that larger/older grasshoppers are not able to support measured resting metabolic rates using only diffusive oxygen delivery (11). As expected, surgical manipulations show that air-flow through these tracheae is more convective in larger/older grasshoppers (11). The increased use of abdominal pumping by larger/older grasshoppers is thought to promote convective oxygen delivery (7).

Any compensatory changes in tracheal structure or function due to possible oxygen delivery limitations should be more pronounced in the areas of the body where thicker cuticular exoskeleton may reduce convective-associated compressions, such as the antennae or legs. Indeed, pumping mechanisms have been found to promote hemolymph flow down blind-ended appendages in many different insect species (14). In promoting hemolymph flow, these pulsatile organs may also help convectively ventilate the tracheae in the appendages and therefore improve oxygen delivery. However, it is unclear whether the number of pulsatile organs or their function is enhanced in larger insects (or older insects).

To promote increased oxygen delivery in the jumping leg, larger/older *S. americana* grasshoppers contain a greater number of tracheoles in the jumping muscle than juveniles (10). The increased tracheolar density throughout the length of the jump-

ing muscle explains why the jumping muscle of larger/older grasshoppers has higher oxygen consumption rates (8). Interestingly, the relative volume of gross tracheae per femoral muscle mass throughout the leg is similar between adults and juveniles (10), suggesting that bulk oxygen flow may be limited down the leg. However, it is possible that the gross tracheae in the legs of older/larger grasshoppers maybe more compressible to enhance convective oxygen delivery down the leg (10), although future studies are needed to address this idea.

ADVANCES IN INSECT RESPIRATORY PHYSIOLOGY: REAL-TIME PHASE-CONTRAST X-RAY

Through the use of the x-ray synchrotron at Argonne National Laboratories, the first real-time x-ray phase contrast studies of living insects have been accomplished (15-17). Synchrotron x-ray phase-contrast imaging allows for objects >10 μm to be observed, which includes most of the gross tracheal structures. Due to the density difference between the air-filled tracheal tubes and the surrounding body tissues, respiratory structures are easily observed within the x-rayed insect (15). The x-rays do not affect the internal temperature, behavior, physiology or survivorship of the insect (16). Further, insects can be manipulated within the x-ray synchrotron beam, so that it is possible to observe and later quantify how internal structures such as tracheae or air sacs respond to atmospheric oxygen changes (17).

Recently our research team has used the x-ray synchrotron to examine the amount of tracheae and air sacs in developing *S. americana* grasshoppers ranging from 10 mg first instars to 2 g adults. Our findings show that adults have both a greater number and individually larger tracheae than juveniles, relative to body size (17). Adults also have a greater number of air sacs than juveniles, especially first instars, which lacked air sacs. Regardless of age, the air sacs had the same ventilatory frequency, as measured by point-counting and video analysis. These findings suggest that the increased convective ventilation of larger/older grasshoppers is due to their greater number of air sacs (17)

We feel that the increased number of tracheal structures in larger/older grasshoppers maybe significant for both their life history and possibly the evolution insect body size. Since adult grasshoppers can both jump and fly, while juveniles can only hop, the increased proportion of respiratory structures to body mass in older grasshoppers could be to support the developing aerobic flight muscles and energetic costs of becoming volant. However, the increased investment in tracheal structure may also suggest that oxygen delivery becomes more challenging for larger insects and may limit body size (17). If larger insects are investing more resources into building respiratory structure and consequentially less in digestive or reproductive tissue, a trade-off may exist that results in a slower growth rate for larger insects and also reduced maximal size (17).

ADVANCES IN INSECT RESPIRATORY PHYSIOLOGY: ELECTRON PARAMAGNETIC RESONANCE

The best way to determine if the oxygen supply is sufficient to meet tissue demands in larger insects is to directly measure the tissue P_{O2}. All studies to date examining the effect of insect body size on oxygen sensitivity or tracheal function have not investigated how body size affects internal P_{O2}. Internal P_{O2} is thought to be a critical signal for many physiological processes, such as molting (18). Attempts at measuring internal P_{O2} in insects are complicated and technically challenging. Traditionally, studies measuring internal P_{O2} have used oxygen electrodes (19); however, these are often too large for small insects and are only useful if the insect's movement is restricted (20).

Recently, electron paramagnetic resonance (EPR) using lithium phthalocyanine (LiPc) crystals has been used for *in vivo* measures of P_{O2} during quiescence and bioluminescence in beetle larvae (21). In this technique, LiPc crystals are injected into the tissue of interest. The crystals are paramagnetic metallophtalocyanines organic compounds with semiconductor properties that make them very sensitive to oxygen (20). LiPc crystals are excellent for *in vivo* biological studies because they have high stability for weeks, low toxicity and short response time (<1 sec) to P_{O2} changes (20).

Our lab has begun to utilize EPR to measure how the internal P_{O2} changes during activity in *S. americana* grasshopper. We measured the internal P_{O2} of the hemolymph surrounding the jumping muscle in adult grasshoppers. Trityl radical-63, a soluble oxygen marker that has the same properties as LiPc crystals, was injected into either the abdomens or femurs of adults to measure hemolymph P_{O2} in those regions at rest and during recovery from two minutes of jumping. Two minutes of jumping is sufficient to show significant rates of fatigue (8).

Our results indicate that the femoral hemolymph has a higher resting P_{O2} than abdominal hemolymph (Figure 1). Although we were not able to jump the grasshopper within the electron paramagnetic machine, we were able to record internal P_{O2} values approximately ten seconds after returning them back into the machine following the two minute jumping trial. During recovery from jumping, the hemolymph P_{O2} in the abdomen is maintained at resting values while the femoral hemolymph P_{O2} rapidly recovers after exercise (Figure 1). This suggests that the increased oxygen levels found in the resting femur were quickly utilized during repeated jumping.

The quick return of the femoral internal P_{O2} to resting values (and slightly above resting values) after recovery from jumping suggests that internal P_{O2} is carefully regulated. The high resting femoral internal P_{O2} and quick recovery in adults may be the result of their increased number of abdominal tracheae and air sacs (7). After jumping grasshoppers, especially larger/older animals, will pump their abdomen to improve convective oxygen delivery (2). Future studies should examine if the internal P_{O2} in the femur during both rest and recovery measured in adults is similar in smaller/younger grasshoppers, especially since their muscles consume proportionally less oxygen during repeated jumping (9) and they have fewer abdominal tracheae and air sacs (17).

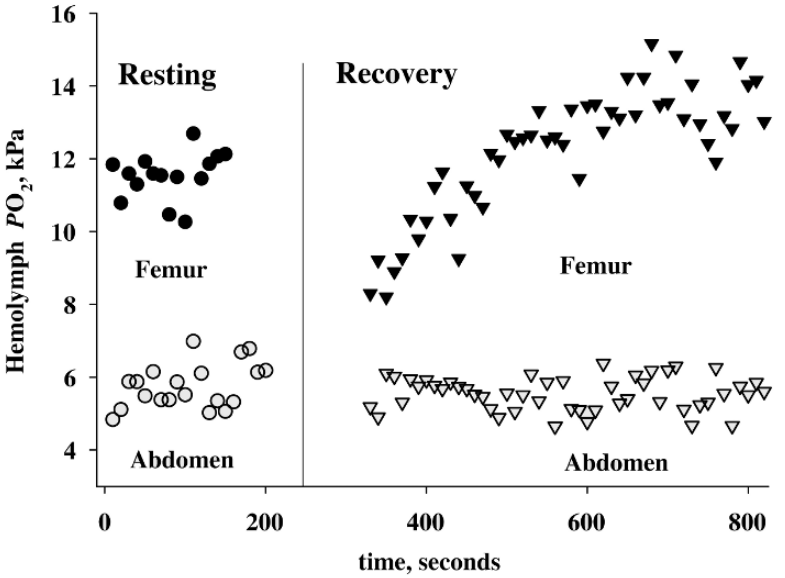

Figure 1. Adult *Schistocerca americana* grasshoppers are not able to maintain a high resting femoral hemolymph internal P_{O2} after two minutes of repeated jumping. Abdominal hemolymph P_{O2} is unchanged by jumping. P_{O2} was measured using an electron paramagnetic soluble oxygen marker.

CONCLUSIONS

Almost all studies to date that have examined the relationship between insect body size and atmospheric oxygen have used a developmental approach to compare larger/older insects to smaller/younger ones. The oxygen sensitivity and performance results have suggested that larger/older insects are not oxygen limited during rest in hypoxia, feeding, or jumping. However, the lack of body size effect on performance or oxygen delivery safety margin may be due to the increased number tracheal structures in larger/older grasshoppers. For example, larger/older grasshopper have more gross tracheal structures in their abdomens and also an increased number of tracheoles in their jumping muscle to promote increased convective ventilation and diffusion of oxygen, respectively. These results suggest that internal P_{O2} may be the driving regulator of tracheal structure and function. Future studies should examine how changes in body size, both through development and interspecifically, affects the regulation and sensing of internal P_{O2} to provide insight into whether oxygen delivery may limit insect size.

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Chapter 18

THE ROLE OF HIF-1 IN HYPOXIC RESPONSE IN THE SKELETAL MUSCLE

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Abstract: During endurance training, exercising skeletal muscle experiences severe and repetitive oxygen stress, and the muscle's ability to cope with and improve its function through that stress is central to its role in the body. The primary transcriptional response factor for hypoxic adaptation is hypoxia inducible factor-1α (HIF-1α), which upregulates glycolysis and angiogenesis in response to low levels of tissue oxygenation. To examine the role of HIF-1α in endurance training, we have created mice specifically lacking skeletal muscle HIF-1α and subjected them to an endurance training protocol. We found that only wild type mice improve their oxidative capacity, as measured by the respiratory exchange ratio; surprisingly, we found that HIF-1α null mice have already upregulated this parameter without training. Furthermore, untrained HIF-1α null mice have an increased capillary to fiber ratio, and elevated oxidative enzyme activities. These changes correlate with constitutively activated AMP-activated protein kinase in the HIF-1α null muscles. Additionally, HIF-1α null muscles have decreased expression of pyruvate dehydrogenase kinase I, a HIF-1α target that inhibits oxidative metabolism. This data demonstrates that removal of HIF-1α causes an adaptive response in skeletal muscle akin to endurance training, and provides evidence for the suppression of mitochondrial biogenesis by HIF-1α in normal tissue.

Key Words: skeletal muscle, endurance exercise, oxidative capacity, HIF

INTRODUCTION

The greatest challenge facing skeletal muscle is the need to match ATP production with energy demand during exercise. As exercise intensity rises, the demand for ATP increases, and more rapid and efficient ways of producing ATP are required. The pathways leading to ATP production during exercise can be divided into two major categories: aerobic (oxygen requiring) and anaerobic (oxygen independent). During exercise, a muscle must balance the input of both aerobic and anaerobic metabolism to meet energy demands, and the balance between the two is determined by the type, intensity, and duration of exercise (5). Endurance exercise relies primarily on aerobic metabolism for ATP generation, meaning the muscle must the available oxygen to produce much-needed ATP. The difficulty of this task is compounded by the availability of

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oxygen to the muscle, which can change greatly from rest to exercise. During exercise in normoxia, the partial pressure of oxygen in the muscle has been measured at 3.1 mm Hg, even though oxygen in the inspired air has a partial pressure of 160 mm Hg, and oxygen in the capillaries in the muscle has a partial pressure of 38 mm Hg (55). This low level of oxygen during exercise necessitates a mechanism to enable the muscle to maintain optimum performance.

THE CELLULAR HYPOXIC RESPONSE AND HIF-1α

The primary oxygen response factors within a cell are the transcription factors of the Hypoxia Inducible Factor (HIF) family, HIF-1, HIF-2 and HIF-3. Only two of these members, HIF-1 and HIF-2, have been characterized appreciably. Of those two, HIF-1 is the more ubiquitous member (67), as the induction of HIF-2 protein under hypoxia is limited to certain cell types within tissues (79).

First purified and sequenced in 1995, HIF-1 is a heterodimeric protein composed of two basic helix-loop-helix-PAS transcription factors: the aryl hydrocarbon nuclear receptor (ARNT, also referred to as Hypoxia Inducible Factor-1β), and HIF-1α (75, 77). While HIF-1α and ARNT are each constitutively expressed and translated, ARNT protein levels are relatively stable but HIF-1α protein levels are regulated primarily by the availability of oxygen to the cell. Under normoxic conditions, HIF-1α protein is hydroxylated by members of a family of prolyl hydroxylases on two conserved proline residues in its oxygen-dependent degradation domain (ODD) (6, 14). This hydroxylation enables recognition of HIF-1α by an E3 ubiquitin ligase complex, of which the von Hippel Lindau (VHL) protein is the primary factor responsible for recognizing and binding to hydroxylated HIF-1α (29, 30). The hydroxylation of HIF-1α at its proline residues is essential for this interaction as their mutation results in less binding of VHL with HIF-1α (14). Further verification of the importance of the proline residues comes from other studies looking at manipulation of the ODD. Wholesale deletion of the ODD results in a stable HIF-1α protein and HIF-1 target gene activation, and fusion of the ODD to a normally oxygen-insensitive protein makes that protein oxygen sensitive (28). The interaction of HIF-1α with VHL results in ubiquitylation of HIF-1α, and targeting of HIF-1α to the 26S proteasome for degradation (9). This regulation of HIF-1α protein through hydroxylation is quite strict; the half-life of new HIF-1α protein under normoxia has been demonstrated to be as short as five minutes (28).

When oxygen concentration drops, and cells and tissues become hypoxic, the hydroxylation of HIF-1α is blocked, resulting in decreased interaction between HIF-1α and VHL (30). As a result, HIF-1α protein is stabilized, allowing it to dimerize with ARNT and turn on transcription of target genes. The oxygen sensing machinery that so tightly regulates HIF-1α under normoxia is quite sensitive to inhibition by hypoxia; hypoxic cells begin accumulating HIF-1α protein within 2 minutes of hypoxic exposure (31). In vivo, the sensitivity of cells to hypoxia is tissue-specific. In work with mice exposed to normobaric hypoxia, Stroka et al. (67) saw that brain tissue begins accumulating HIF-1α protein when inspired oxygen is dropped to 18%, while kidney and liver

only respond to more severe hypoxia. Additionally, the authors found stable HIF-1α protein under normoxia in skeletal muscle, showing that some tissues have the ability, and need, to accumulate HIF-1α protein independently of hypoxia. This finding was recently repeated by Pisani and Dechesne (54), who additionally showed that normoxic HIF-1α stability in the muscle is dependent on fiber type. Muscles that are composed primarily of type II fast twitch fibers have a higher level of HIF-1α protein at rest in normoxia than muscles with a higher proportion of type I fibers.

Once HIF-1α is stabilized, it interacts with ARNT, forming the HIF-1 complex. This enables HIF-1 to recognize hypoxia responsive elements (HRE) in the promoters and/or enhancers of genes in the nucleus. The HRE is a short consensus sequence that HIF-1 binds to in order to upregulate transcription of target genes (44, 76). Once activated, the transcriptional response of HIF-1α to hypoxia enables cells to cope with oxygen stress while working to increase oxygen delivery (65). To help cells and tissues survive oxygen stress, HIF-1α upregulates transcription of genes that amplify glycolysis and glucose transport into the cell. Genes in this category include glucose transporters 1 and 3 (GLUT1, GLUT3), as well as the glycolytic genes hexokinase I and II (HKI, HKII), phosphoglycerate kinase 1 (PGK1), and lactate dehydrogenase A (LDHA), among others (64). In order to increase oxygen availability, HIF-1α coordinates a response that increases oxygen delivery to the hypoxic region. Two key transcriptional targets for this function are vascular endothelial growth factor (VEGF), and erythropoietin (EPO) (64). Other HIF-1α target genes include genes involved in cell cycle and apoptosis signaling, however, the role of HIF-1α in the cellular proliferation/survival response is not completely understood. In addition to the multitude of genes identified as having HREs in their promoters (meaning they can be directly regulated by HIF-1α), many more genes have been shown to have expression patterns correlating with HIF-1α activity, indicating that they are also directly or indirectly regulated by HIF-1α. GLUT4, the primary muscle glucose transporter, falls into this category (66). New HIF-1α targets are continually being discovered as the understanding of how HIF-1α helps cells and tissue respond to hypoxia grows.

Our research has shown that loss of HIF-1α can have profound effects on cells and tissues. The primary result of the loss of HIF-1α is that cells are unable to upregulate HIF target genes in response to hypoxia. This leads to a failure to upregulate glucose transport and glycolysis, resulting in decreased ATP levels during hypoxia (63). Surprisingly, this failure extends to normoxia as well for some cell types, as macrophages lacking HIF-1α have as little as 15-20% of the ATP content under normoxic conditions as control macrophages (10). HIF-1α is also essential for development, where local hypoxia results from the lack of an established vascular system. In evidence of this, mice lacking HIF-1α in their germ line die *in utero* due to defects in cephalic vascular formation and defective neural fold formation (58).

Another important role for HIF-1α has been found in tumor growth and development. Solid tumors become hypoxic as they grow larger, and tumors forming following inactivation of the VHL tumor suppressor protein are aggressive and well vascularized (38), leading to the hypothesis that HIF-1α is a positive factor in tumor development. To that end, we have shown that solid tumors lacking HIF-1α do not grow as rapidly as normal tumors, indicating that this is indeed the case (59). Furthermore, we recently

found that deletion of HIF-1α in mammary epithelial tissue results in delayed tumor onset, retarded tumor growth, and reduced pulmonary metastasis in a breast cancer model system (39).

Tissue and cell type-specific deletion of HIF-1α has shown HIF-1 signaling to be integral in many different places in the body. Deletion of HIF-1α in chondrocytes results in bone deformities and abnormalities in the trachea due to increased chondrocyte growth (62), while in myeloid cells, loss of HIF-1α reduces their mobility and invasiveness, and their ability to kill bacteria (10). Combining the results of these studies shows that the hypoxic response through HIF-1α plays an important role in development, disease, and homeostasis.

MUSCULAR RESPONSE TO ACUTE ENDURANCE EXERCISE

As referenced above, skeletal muscle experiences a drop in intramuscular oxygen during exercise, leading to a hypothesis for a possible role for HIF-1α in the muscle during and following exercise. Surprisingly, however, little research has been done looking directly at HIF-1α function in the muscle prior to our studies.

In the muscular response to exercise, several changes occur that are likely mediated by HIF-1α. Due to the increased demand for oxygen in the muscle, both the body and the skeletal muscle undergo several acute performance-oriented changes. These changes have the goal of increasing oxygen delivery to the muscle and improving its metabolic capabilities. Since an acute exercise bout is too short of a time period to allow for vascular remodeling or a significant increase in red blood cell content, one of the primary ways exercising skeletal muscle receives greater oxygen delivery during exercise is through increased blood flow to the skeletal muscle. This is accomplished through two main pathways: a decrease in blood flow to non-exercising tissues (i.e., the kidney and spleen) and increased blood flow to the skeletal muscle itself (56). In addition to increased oxygen delivery, the greater blood flow also allows for increased metabolite delivery to and waste clearance from the exercising muscle.

The metabolic changes in exercising muscle serve to increase ATP production while minimizing the impact of non-essential ATP consuming pathways. A key protein that helps the muscle accomplish this is the AMP-activated protein kinase (AMPK). Exercise, and the resulting increase in ATP consumption, causes an increase in the AMP to ATP ratio. AMP then binds with AMPK, making AMPK a better substrate for phosphorylation and activation by an upstream kinase (23). Once activated, AMPK phosphorylates targets leading to increased glucose transport, glycolysis, and fatty acid oxidation, as well as decreased ATP consumption (80). Two key phosphorylation targets are the GLUT4 Enhancer Factor (GEF) and Acetyl-CoA Carboxylase (ACC). Phosphorylation of GEF by AMPK results in an increase in GLUT4 expression and, eventually, increased GLUT4 protein accumulation (25, 85), while phosphorylation of ACC inactivates it and causes a decrease in malonyl-CoA levels (33, 82). Malonyl-CoA inhibits carnitine palmitoyltransferase (CPT), which catalyzes a rate-limiting step of fatty-acid β-oxidation (81). The AMPK-caused decrease in malonyl-CoA allows for an increase in CPT activity, thus increasing β-oxidation during exercise. Loss of AMPK

in the skeletal muscle, through the use of a dominant negative form of AMPK's catalytic α subunit, results in the muscles being more sensitive to, and slower to recover from, fatigue (46), and demonstrates the importance of AMPK during exercise.

Additional changes in the muscle during exercise directly affect glycolytic flux, an area that may be mediated by HIF-1α activity. Glucose uptake by the muscle increases dramatically during exercise (37), which is likely a result of increased glucose transporter 4 (GLUT4) translocation to the cell surface (71). Additionally, glycolytic flux is constant and integral during aerobic and anaerobic exercise, and leads to lactate accumulation during both (35).

As can be expected, mutations that block or inhibit steps in these important metabolic pathways can have dramatic phenotypes. Several myopathies have been characterized that result from a blockage in carbohydrate metabolism, and are collectively referred to as glycogen storage diseases (GSD). Two of these diseases are GSD V, muscle glycogen phosphorylase deficiency, and GSD VII, muscle phosphofructokinase deficiency, also known as McArdle's Disease and PFKD, respectively. Patients with either myopathy have decreased carbohydrate utilization resulting in increased glycogen storage, decreased lactate accumulation during exercise, exercise intolerance, and muscle damage following intense exercise (13). As a result of the decreased carbohydrate metabolism, the myopathic muscles frequently have a compensatory response, resulting in their relying more on phosphocreatine and/or aerobic metabolism for ATP production during exertion (2, 73). Another compensatory response, especially in patients with McArdle's Disease, is the Second Wind phenomenon. In this case, normally exercise intolerant patients perform an initial exercise with difficulty, rest briefly, and can then exercise for a significantly longer period of time with much less discomfort. The cause of this phenomenon is not fully understood, but is likely due to compensation from blood glucose and increased fatty acid oxidation (21).

In addition to maintaining ATP levels, another main challenge of skeletal muscle is resisting fatigue. Muscle fibers, and therefore muscles with differing fiber composition, vary in their resistance to fatigue. Type I fibers, which are slow twitch and highly oxidative, are highly fatigue resistant. On the other hand, fast-twitch type II fibers are more susceptible and fatigue quite rapidly. The mechanisms leading to fatigue sensation are not completely understood yet, but to a large degree, are thought to involve lactate signaling. As exercise continues, serum lactate levels increase, and lactate has been shown to correlate well with fatigue sensation. One classic experiment by Fitts and Holloszy (16) demonstrated that muscle contractile force decreases as lactate levels increase. Additionally, administration of dichloroacetate, an activator of pyruvate dehydrogenase (PDH) through inhibition of pyruvate dehydrogenase kinase, decreases lactate accumulation and increases endurance capacity in untrained subjects (41). However, as this process involves PDH activation, and thus will increase oxidative metabolism, the decreased lactate accumulation may merely be correlative to the increase in endurance rather than causative. Other causes of fatigue may be intracellular changes, such as changes in pH, decreased ATP levels, or a failure to regulate Ca^{2+} release or reuptake (12). Since the HIF-1α mediated increase in glycolysis also results in increased lactate production, modulation of HIF-1α in the muscle may have an impact on endurance and fatigue.

Gene transcription in the skeletal muscle is greatly affected both during exercise and recovery following exercise. Expression of interleukin 6, a cytokine that has been proposed to have a large role in fatigue sensation, is markedly increased during exercise (34). The transcription of several important metabolic genes is affected by exercise. In a study looking at gene expression immediately following a four hour cycling exercise in untrained patients, Pilegaard et al. (53) saw elevated expression of heme oxygenase-1 (HO-1) and pyruvate dehydrogenase kinase 4 (PDK4). During the recovery from exercise, muscles further increased PDK4 expression, and also upregulated hexokinase II (HKII), lipoprotein lipase (LPL), and uncoupling protein 3 (UCP3). In a different study, and of specific relation to HIF-1α, expression of VEGF, and its receptor Flt-1 were seen to be upregulated following exercise in rats (51). Additionally, untrained skeletal muscle has a marked upregulation of HIF-1α, HIF-2α, and EPO mRNA during recovery from exercise (1, 42). These transcriptional changes show a coordinated effort by the muscle to adapt to the stress of exercise and become better suited for endurance activities, and also give further evidence for an important role for HIF-1α function in the muscle.

The role of HIF-1 in untrained muscle and during acute exercise has been studied, although its function is not yet completely understood. As mentioned above, resting untrained skeletal muscle has stable HIF-1α protein, suggesting that HIF-1 has an important role in maintaining homeostasis in the muscle. This hypothesis was strengthened by the findings of Ameln et al. (1), who recently showed that acute exercise leads to increased stabilization of HIF-1α protein, perhaps giving the mechanism for the increase in expression of HIF-1 target genes following exercise. However, these earlier studies still did not elucidate the role HIF-1 plays in the way muscles respond during exercise.

With this question in mind, we sought to determine the exact role of HIF-1 signaling in untrained skeletal muscle utilizing a tissue-specific knockout mouse. By crossing mice with LoxP flanked alleles of HIF-1α (59) with mice expressing the Cre recombinase transgene under the control of the muscle creatine kinase promoter (MCK-Cre mice) (7), we were able generate mice lacking HIF-1 signaling in the skeletal muscle (45). Surprisingly, the skeletal-muscle HIF-1α null mice had normal morphology of their muscles, and isolated stimulation of gastrocnemius muscles and single fibers revealed similar force generation, Ca^{++} release, and fatigue rates in control (WT) and HIF-1α null (HIF-null) muscles. However, during these contractions, HIF-null muscles had to rely more heavily on phosphocreatine for ATP generation, and had difficulty maintaining ATP levels. Additionally, the HIF-null muscle accumulated more early glycolytic metabolites, indicating that loss of HIF-1α impeded glycolytic flux in the muscles. Analysis of muscles from mice following a controlled run confirmed this, as HIF-null muscles failed to upregulate expression of key glycolytic enzymes, and were also unable to maintain enzymatic activity of PFK. Correlating with this, the HIF-null mice accumulated less lactate in their serum during the run.

Surprisingly, these changes added up to an increase in endurance for the HIF-null mice when the mice were subjected to swimming and uphill running tests. Further analysis of the HIF-null muscles revealed that loss of HIF-1α lead to an increase in β-hydroxyacyl-CoA dehydrogenase and citrate synthase, indicating increased aerobic ca-

pacity in these mice and contributing to the increase in endurance. Unfortunately, this was not a win-win situation for the mice, as loss of HIF-1α resulted in increased muscle damage following the endurance test. Additionally, when the mice were forced to run downhill, an eccentric exercise that forced the muscles to rely on glycolytic metabolism (48), the HIF-null mice lost their endurance edge due to their impeded glycolytic flux.

From this study, and earlier results, it can be seen that loss of HIF-1α in the skeletal muscle causes an adaptive response leading to an increased capacity for endurance exercise. It can also be seen that HIF-1α is necessary for the maintenance of optimal glycolytic flux in the skeletal muscle. Finally, given the increased muscle damage seen in the HIF-null muscle, it is tempting to speculate that HIF-1α is essential for proper sensation of fatigue, and preventing injury to the muscle from overexertion.

MUSCULAR RESPONSE TO ENDURANCE TRAINING

The ability of the skeletal muscle to acclimate to repeated exertion is central to its role in the body. This ability to acclimate enables it to become better suited and prepared for exercise, something muscle can achieve rather quickly. Endurance training studies have been carried out extensively in humans as well as animal models to understand how muscles undergo this acclimation to exercise. Two main categories that the changes fall under are morphological changes and enzymatic changes, resulting in a change in the profile of the muscle. The end result of endurance training is that the skeletal muscle has improved delivery and utilization of its available oxygen, leading to enhanced performance and endurance. Given that oxygen is central to these changes, it is very likely that the primary hypoxia responsive factor, HIF-1, has a large role in helping the muscle to acclimate to repeated exercise.

The most significant change seen in the muscle as a result of endurance training is increased endurance. However, there are other markers of improved muscle capability beyond just endurance. Two of the more prominent ones are the respiratory exchange ratio (RER) and VO_2max. A measure of fuel utilization, the RER generally has a downward shift following training, indicating an increase in fatty acid oxidation relative to carbohydrate metabolism. VO_2max is the maximal oxygen consumption achievable by the subject, and is closely linked to aerobic metabolic capacity. Like overall endurance, this parameter also usually increases following endurance training, indicating an increase in oxidative capacity by the subject.

Morphologically, there are two main adaptations a muscle undergoes during endurance training – an increase in capillary density and a shift in fiber type composition. The advantage of increased capillary density is obvious as it allows for increased oxygen and metabolite delivery to the exercising muscle, thus increasing aerobic capacity. Increased capillary density can occur after only six to eight weeks of endurance training; this short of a period has been shown to lead to a 30% increase in capillary density (27). The HIF-1α target, VEGF, is of critical importance here as deletion of VEGF in the muscle following development results in a dramatic drop in muscle capillary density and capillary to fiber ratio (68).

The shift in fiber type composition allows the skeletal muscle to better take advantage of this increase in oxygen delivery, and also contributes to the changes in VO_2max and RER that are seen in trained patients and animals. In addition to the two main categories (type II fast twitch and type I slow twitch), muscle fibers can be classified according to their metabolic preferences. Type I fibers are oxidative, and rely heavily on aerobic metabolism, while type II fibers can be broken into two major categories: type IIA and type IIB. Type IIB fibers are largely glycolytic, while type IIA fibers are largely oxidative despite being fast-twitch. Endurance training has been shown to cause a shift toward slow twitch fibers in humans (17). Additionally, trained muscles have a greater percentage of type IIA fibers versus type IIB, indicating an increase in oxidative capacity (24). This shift toward an oxidative profile enables a trained muscle to take full advantage of the increased capillary density.

In addition to morphological changes, there are numerous metabolic changes in trained muscle relative to untrained muscle. Generally, these changes increase the muscle's ability to rapidly produce ATP during exercise, especially from the beta-oxidation of fatty acids. Improvements in ATP production generally come in the form of upregulated metabolic enzymes and the resulting increased capacity for oxidative phosphorylation. Increased oxidative phosphorylation is a result of elevated mitochondrial density in the muscle, and upregulation of levels of the metabolic enzymes contained therein. In previous studies, endurance training has resulted in an increase of 40% in mitochondrial volume in the skeletal muscle, and significant increases have also been seen in the aerobic metabolic enzymes citrate synthase, β-hydroxyacyl-CoA dehydrogenase, and carnitine palmitoyl transferase (4, 22, 27, 61).

Oxidative phosphorylation is not the only metabolic pathway upregulated as a result of training. Activity of hexokinase, a HIF-1 target, also increases as a result of endurance training, indicating improved carbohydrate metabolism (69). The benefit of this increase for the muscle is two-fold. First, as the initial enzyme in glycolysis, an increase in hexokinase activity will allow for greater flux into glycolysis, allowing for greater pyruvate and ATP production. Secondly, since muscle lacks glucose-6-phosphatase, any glucose that enters the muscle will be phosphorylated by hexokinase and remain in the muscle to either be metabolized immediately or stored as glycogen for later use. An increase in hexokinase will thus help ensure there will be enough carbohydrate fuel for the muscle during exercise. In fact, hexokinase activity can control exercise endurance in a dose-dependent manner; in genetic mouse models, increased hexokinase activity was seen to correlate quite well with increased endurance (19).

A third metabolic consequence of endurance training is an increase in glycogen storage in the muscle. This is not only a result of the increased in hexokinase activity, but also a result of increased glycogen synthase (8), and is another way in which a trained muscle is better prepared for exertion. Additionally, endurance-trained muscle is slower to deplete its glycogen stores than untrained muscles, a change which enables muscles to perform longer since they can spare glycogen for when it is absolutely needed (24).

Although not yet completely understood, the mechanism underlying the acclimation of skeletal muscle to endurance training is coming to light, and some of the key factors regulating the response to endurance training have been identified. Surprisingly,

despite the preponderance of HIF-1α targets following exercise, and the importance of angiogenesis to the training response, much of the research into factors regulating the endurance training response has focused on other genes. Two important transcription factors that have a role in upregulating oxidative metabolism are the nuclear respiratory factors 1 and 2 (NRF-1 and 2). NRF-1 and 2 bind to specific response elements of target genes such as mitochondrial transcription factor A (TFAM), cytochrome c, and succinate dehydrogenase subunit B (60). Highlighting the importance of NRF-1, endurance exercise has been shown to increase NRF-1 protein, and a mouse constitutively over expressing NRF-1 has increased oxidative capacity, as well as increased GLUT4 expression (3). However, the NRF-1 transgenic mouse does not have elevated citrate synthase, cyclooxygenase-IV, or succinate:ubiquinol oxidoreductase, indicating that NRF-1 by itself is not sufficient to cause the training-induced changes. Very little research has been done on a connection between HIF-1 and NRF-1, although the two have parallel expression patterns in postnatal hearts (50).

Members of the peroxisome proliferator-activated receptor (PPAR) family have also has been hypothesized to have a role in the muscular response to training. One of them, PPARα, has been shown to upregulate mitochondrial genes in charge of fatty acid oxidation, leading to increased oxidation (20, 72). The primary member of the PPAR family in the skeletal muscle is PPARδ, which has been shown to have an important role in determining muscle oxidative capacity. In work with a PPARδ transgenic mouse, Wang et al. (78) showed that overexpression of PPARδ in the skeletal muscle results in a mouse with a greater proportion of type I oxidative fibers, leading to increased mitochondrial content, resistance to obesity, and dramatically increased endurance. Intriguingly, hypoxia has been shown to down-regulate PPARα, and this down-regulation appears to be HIF-1 dependent (49). It is not currently known if this down-regulation extends to PPARδ as well, but the HIF-1 regulated gene DEC1/Stra13 has been shown to inhibit PPARγ-2 (84). These findings make it interesting to speculate as to whether HIF-1α has a similar interplay with other members of the PPAR family, in particularly PPARδ.

Another gene that has been shown to possibly have a role regulating the muscle response to endurance training is PPARγ coactivator-1α (PGC-1α), which stimulates the expression of NRF-1 and NRF-2, among other genes (83). In the same study, Wu, et al. also saw that PGC-1α binds with NRF-1 and coactivates it at the TFAM promoter, leading to increased mitochondrial biogenesis. In the skeletal muscle, PGC-1α is normally expressed in type I fibers, and constitutive expression of PGC-1α in the muscle at normal physiological levels results in a transition of type II fibers to being more like type I fibers. This results in the fibers becoming more fatigue resistant in isolated stimulation assessments (40).

These three families of genes, the NRFs, PPARs, and PGCs, all have the potential to regulate the changes seen in the muscle. They all increase oxidative capacity and improve muscle performance. Interestingly, not much research has been done on any connections between them and HIF-1, even though the demands of exercise, which induce HIF-1α, are also what lead to their activation, either at the protein level or through transcription (47, 57, 70).

In part because of the repeated oxygen stress placed on skeletal muscle during ex-

ercise, a role for HIF-1α in the muscular response to exercise and training has been proposed (18, 26). Some of the responses seen from muscle during training further corroborate this hypothesis. As hexokinase II and VEGF are two prominent HIF-1α targets, and since an increase in hexokinase and angiogenesis are two common changes following training, a role for HIF-1α can be proposed. Additionally, training under ischemic conditions results in greater citrate synthase activity than exercise with normal blood flow (15, 32). Finally, as mentioned before, transcription of several HIF-1α targets is increased in the muscle following exercise, and transcription of HIF-1α itself is upregulated following repeated hypoxic exercise (74). Thus it can be hypothesized that HIF-1α has a role in the muscular training response to exercise, although no research had directly addressed this prior to our work.

In order to address the role of HIF-1α in skeletal muscle during endurance training, we subjected WT and HIF-null mice to a training protocol. Surprisingly, both genotypes responded equally well to endurance training. Analysis of muscles from mice following training revealed that WT mice were able to "catch up" to HIF-null mice in the areas in which loss of HIF-1α had caused an adaptive response in the muscle. These areas included aerobic metabolism, mitochondrial DNA content, and capillary to fiber ratio. The adaptive response in these parameters in the HIF-null mice was sufficient to endure the training stimulus, and thus no further changes were seen in these parameters. Also consistent with trained muscle, AMPK activation was increased in resting HIF-null muscle, indicating that AMPK signaling has a role in the adaptive response seen in the HIF-null muscles. Hexokinase activity increased in trained muscles of both genotypes, indicating that hexokinase made a strong contribution to the increases in endurance seen, consistent with previous studies (19, 69).

These results, and our work with untrained skeletal muscle, contrast starkly with the hypothesis that HIF-1α plays an integral role in the muscular response to endurance training. Thus, it appears that removing HIF-1 signaling has predisposed the skeletal muscle for endurance training, leading to the speculation that one aspect of endurance training may, in fact, be to remove HIF-1 signaling. Several lines of evidence support this hypothesis. Recent studies have shown that HIF-1α has a suppressive effect on oxidative metabolism; Dahia et al. (11) have shown reduced succinate dehydrogenase subunit B protein levels in response to constitutive HIF-1α activation, and two studies have demonstrated that HIF-1α upregulates pyruvate dehydrogenase kinase I, an inhibitor of pyruvate dehydrogenase and oxidative metabolism (36, 52). Our results corroborate this, as cultured myoblasts lacking HIF-1α have reduced PDK1 protein and increased oxygen consumption in response to hypoxia. Additionally, resting HIF-null skeletal muscle has reduced PDK1 mRNA, something that WT muscle achieves following endurance training.

Thus it is now apparent that HIF-1α signaling actually is inhibitory to endurance training. In keeping with the revised hypothesis that endurance training has a result of removing HIF-1α signaling, Lundby et al. (43) recently demonstrated that induction of HIF-1α mRNA is significantly reduced in trained muscle from human subjects relative to untrained muscle following exercise. While HIF-1α is important for optimal muscle function during acute exercise, it is non-essential for, and likely inhibitory of, endurance training.

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Chapter 19

GENE EXPRESSION IN WORKING SKELETAL MUSCLE

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Abstract: A number of molecular tools enable us to study the mechanisms of muscle plasticity. Ideally, this research is conducted in view of the structural and functional consequences of the exercise-induced changes in gene expression. Muscle cells are able to detect mechanical, metabolic, neuronal and hormonal signals which are transduced over multiple pathways to the muscle genome. Exercise activates many signaling cascades - the individual characteristic of the stress leading to a specific response of a network of signaling pathways. Signaling typically results in the transcription of multiple early genes among those of the well known fos and jun family, as well as many other transcription factors. These bind to the promoter regions of downstream genes initiating the structural response of muscle tissue. While signaling is a matter of minutes, early genes are activated over hours leading to a second wave of transcript adjustments of structure genes that can then be effective over days. Repeated exercise sessions thus lead to a concerted accretion of mRNAs which upon translation results in a corresponding protein accretion. On the structural level, the protein accretion manifests itself for instance as an increase in mitochondrial volume upon endurance training or an increase in myofibrillar proteins upon strength training. A single exercise stimulus carries a molecular signature which is typical both for the type of stimulus (i.e. endurance vs. strength) as well as the actual condition of muscle tissue (i.e. untrained vs. trained). Likewise, it is clearly possible to distinguish a molecular signature of an expressional adaptation when hypoxic stress is added to a regular endurance exercise protocol in well-trained endurance athletes. It therefore seems feasible to use molecular tools to judge the properties of an exercise stimulus much earlier and at a finer level than is possible with conventional functional or structural techniques.

Key Words: exercise, molecular, morphology, mitochondria

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PHENOTYPIC PLASTICITY OF MUSCLE STRUCTURE AND FUNCTION

Human exercise performance capacity varies widely. While most healthy young male subjects are able to pedal a bicycle ergometer at 200 Watts for 15 to 20 minutes the best of human athletes can maintain just over 500 Watts for one hour (24). This stunning feat is the consequence both of athletic endowment (genetically determined) as well as years of highly specific exercise training. The extraordinary performance of endurance athletes, such as cyclists, is due to many concerted adaptations of the pathway for oxygen from lungs to skeletal muscle mitochondria (17). On the level of skeletal muscle tissue, we see in highly trained endurance athletes a much larger capillarity (2.2 capillaries per muscle fiber, than in untrained subjects of similar age (1.1 C/F; (38) Likewise, the mitochondrial content of trained skeletal muscle fibers also differs by more than two-fold between world class athletes (11.4 percent of the muscle fiber volume, Vvmito) and untrained subjects (4-5 percent Vvmito; (14) . Interestingly, a similar two-fold difference can be found between athletes and sedentary subjects with regard to their intramyocellular lipid content (IMCL; (14). Not only do we find remarkable structural differences between muscle samples of athletes and sedentary subjects in cross-sectional studies, it is amply demonstrated that skeletal muscle tissue can rapidly change its oxidative capacity when a training regimen is implemented in previously sedentary subjects. Typically, mitochondria and capillaries can increase by approximately 30% with 6 weeks of endurance exercise training (15). Endurance training can be characterized as “low- load, high-repetitive” exercise. In a typical one- hour training session, muscles are loaded up to 5000 times with as little as 10 to 15% of their maximal voluntary contraction force (MVC). Strength training by contrast is typically “high-load, low-repetitive”. A single muscle, or rather a functional muscle group, may experience only 10 to 12 contractions per training session, but those contractions are near maximal, very close to MVC. In power-lifting we find even larger differences between trained and untrained individuals as seen in endurance exercise. While most of us are able to clear some 80kg on a bench-press, the current world record for this exercise is 457 kg (Scot Mendelson). Likewise, improvements in muscle strength with appropriate training occur over similar time courses and with similar gains both structural and functional as those seen with endurance exercise training (28; 33).

In the context of the current review we would like to exploit the fact that both endurance and strength training protocols have been modified with the aim to optimize training stimuli. In endurance exercise, hypoxia (equivalent to altitudes of 1800 to 4000m) during training sessions has been used with the rationale that local muscle tissue hypoxia is an important signal for metabolic muscle adaptations and that systemic hypoxia should increase this signal (16). In strength training emphasis has been on the use of eccentric contractions (i.e. activation of muscles during lengthening) to enhance the effect of training regimens (9; 34). Eccentric contractions have a number of particular properties. At higher given angular velocities, peak torque in eccentric contractions can exceed peak torque in concentric contractions by more than two-fold (7), putting muscles at risk of damage and leading to delayed onset muscle soreness (DOMS; (21).

At similar force developments eccentric contractions are performed with substantially lower electromyographic activities (indicating the recruitment of fewer motor units) and hence have up to four-fold lower energy requirement (2). The lower energy requirements of eccentric contractions result in massively reduced physiological responses of the cardiovascular system to a given mechanical exercise load. This interesting feature of eccentric exercise has been exploited in exercise paradigms for people with cardiovascular limitations in order to maximize mechanical stress on muscle tissue at low metabolic costs (23). The clinical relevance of this property of eccentric exercise stems from the observation that repeated continuous mild eccentric exercise results in gains in muscle force and cross-sectional area similar to those seen after classical high-load concentric strength training (19).

The purpose of the present review is to analyze the classical training paradigms of endurance and strength training as well as their modifications discussed above with regard to their molecular signature. We suggest that gene-expressional analyses should allow for a very detailed evaluation of the effect of training interventions, much finer than functional tests or structural analyses. Current functional laboratory tests for athletes estimate relevant performance variables with error margins of a few percent, while differences of race times between the winner and the runner-up are typically at least one order of magnitude smaller. It is thus currently not possible to identify winners with lab tests. This analogy is not to say that molecular tools will eventually allow us to identify winners. The analogy indicates however, that functional tests currently used as "gold standard" are very coarse global assessment tools that carry virtually no information as to the fine biological adjustments that enable an organism or subject to perform the particular function in question.

THE MOLECULAR BASIS OF MUSCLE PLASTICITY

The adoption of molecular techniques for the study of muscle adaptation in the early 1990s gave access to studies aimed at delineating the mechanisms underlying muscle plasticity. From animal work using chronic electrical stimulation it appeared that pretranslational events had a major share in activity-related changes in enzyme activities (see (4). A particular challenge of early work in this area was to develop technical protocols that worked for small (mg) samples of muscle tissue obtained through biopsies and capable of reliably ascertaining differences in RNA concentrations of less than two-fold. We used quantitative PCR to look at biopsies (vastus lateralis muscle) of highly trained endurance runners and sedentary controls differing 2-fold in VO_2max and 1.9-fold in total muscle mitochondrial volume (26). We found the expression of all six investigated RNAs coding for enzymes of oxidative phosphorylation to be increased in direct proportion to the higher mitochondrial content of the muscle. Our data furthermore suggested pretranslational mechanisms to be responsible for the increase in nuclear encoded mitochondrial transcripts. By contrast, mitochondrially encoded RNAs were found to be increased as a consequence of an increase in mitochondrial DNA. A recent review of Hood (13) a key figure in research on mitochondrial biogenesis, confirms these early findings and presents the current view of the coordination of

transcription of two genomes, synthesis of proteins and lipids as well as the assembly of multisubunit protein complexes which is characterized morphologically as "increase in mitochondrial volume". With an elegant experiment using nuclear run-ons on human muscle biopsies at various time points after exercise Pilegaard et al.(25) provided the direct evidence that a transient increase in transcription rate was followed by an increase in mRNA of several metabolic genes. They also showed that mRNA concentrations remained elevated beyond 22 hours when exercise was carried out repeatedly over several days. This was taken to suggest that transient increases in transcription during recovery from repeated exercise result in a gradual accumulation of mRNA, thus representing the basic mechanism of muscle cellular adaptation to increased contractile activity.

Having established that an important mechanism of muscle plasticity is the accretion of mRNA through repeated transient increases of transcription of metabolic and presumably of structure genes – it remains to be elucidated how the muscle cell senses the specificities of exercise related phenomena and uses the resulting signals to control gene expression. From common sense functional considerations it seems clear that a muscle cell must be able to sense a number of different stimuli. General molecular principles indicate that these initial queues are then transmitted over signaling cascades resulting in activation of a number of transcription factors. In turn, these act on early response genes (such as fos or jun and many others) that influence downstream targets. Alternatively, some transcription factors may directly affect downstream "structure" genes. We have proposed that under conditions of exercise muscle cells are subjected to at least four different important stressors: mechanical load, metabolic disturbance, neuronal activation and hormonal alteration (10) gives a coarse overview of known key factors that modulate the response to these stressors in muscle cells and their relationship (Fig 1). Note that with any type of exercise all stressors will always be active to some degree; however, depending on the type of exercise individual stressors will predominate. In strength-type exercise the dominant stressor is mechanical load while in endurance exercise metabolic disturbance is more important.

Mechanical load is thought to act primarily through integrins and integrin-associated signaling pathways, (see (5). Integrins serve as the link between extracellular matrix and cytoskeleton and are therefore critical to sensing of external mechanical events. In this context the formation of focal adhesion complexes is critical for the cellular transduction of a mechanical signal such as stretch of skeletal muscle fiber (11; 27).

Metabolic disturbances such as shifts in pH, temperature, oxygen tension and energy status within the muscle cell are key feature of muscle work. A central role in metabolic sensing has been assigned to the AMP activated kinase, AMPK, (12). AMPK is implicated in regulation of substrate metabolism and mitochondrial biogenesis, and via inhibition of the TOR pathway in muscle impedes protein metabolism and hence muscle hypertrophy (3).

Neuronal activation is a prerequisite for normal muscle contraction activity. Fluctuations in intramyocellular Ca^{2+} levels are decoded for amplitude and frequency and linked to gene expression by Calmodulin dependent Kinases (CaMK). While CaMKII seems to be involved in regulating oxidative enzyme expression, mitochondrial biogenesis and fiber type specific myofibrillar protein expression, the role of CaMKIV

remains more elusive (6).

Hormonal alterations both local and systemic are inevitable consequences of any type of exercise activity. Androgens, growth hormone, IGF-I and its splice variants, Insulin and Vitamin D positively affect muscle growth/volume mostly through their activation of satellite cells. By contrast, satellite cell activation is repressed by myostatin, glucocorticoids, TNF and IL-1 and IL-6 (30).

While typical training paradigms for strength and endurance are well established – and the functional and structural outcome of these training protocols is well defined and relatively stereotyped; the molecular machinery that intervenes between the stimulus and the outcome is of bewildering complexity. There is massive crosstalk between the different pathways, many factors are sensitive in the time domain and important players might not yet be discovered. There is considerable more research needed to understand training phenomena in muscle on the mechanistic level – and much more when training is seen as a system function of an organism with other organs such as the heart, the brain, the kidney and the liver contributing significantly to the overall training response.

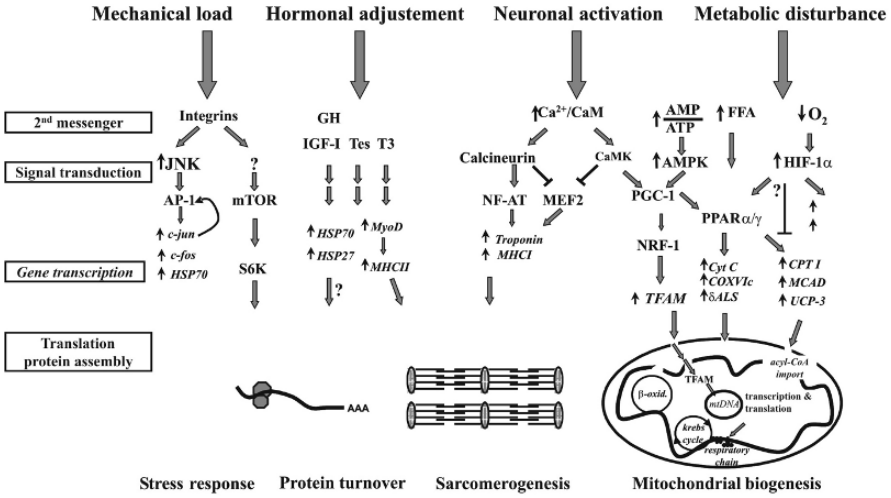

Figure 1. Schematic representation of influence of main stressors of muscle tissue on gene expression in skeletal muscle tissue. Only selected pathways are presented. During exercise all stressors are active – but differ in importance depending on the quality of the exercise stimulus (adapted from (10).

THE MOLECULAR SIGNATURE OF TRAINING IN HYPOXIA

As indicated above we have been interested in the effect of hypoxia on working muscle with the aim of modifying a metabolic stressor presumed to be important for muscle adaptations in endurance type exercise. While the advantage of "training high

– living low" in terms of improvement of athletic performance capacity (at altitude or at sea level) has remained disputed (1; 20) this type of training has been shown to have some specific physiological effects which are coherent with molecular changes observed at the muscle tissue level.Vogt et al (36) using quantitative PCR have shown the steady state levels of mRNA for hypoxia inducible factor 1 (HIF-1α) to be increased in subjects trained for six weeks in normobaric hypoxia (equivalent to an altitude of 3850m) when compared to subjects working at similar relative workloads in normoxia. This important finding of an up-regulation of the key transcription factor HIF-1α with training in hypoxia has been replicated by (37) who found HIF-1α mRNA to be up-regulated in 9 well trained endurance athletes who had a short hypoxia stress added to their regular training schedule. While it has been difficult to demonstrate clear-cut improvements in accepted physiological descriptors of aerobic performance capacity such as VO_2max (8) ; the addition of hypoxia to endurance exercise stress leaves a prominent signature on a number of genes involved in key regulatory functions of hypoxia adaptation (37). Key transcripts of carbohydrate metabolism (Glut-4,glucose transporter 4 ; PFKm, 6-phosphofructokinase muscle type), mitochondrial biogenesis (PGC1α, peroxisome proliferator activated receptor; Tfam, mitochondrial transcription factor 1) and mitochondrial metabolism (CS, citrate synthase; COX-1, cytochrome oxidase subunit 1; COX-4, cytochrome oxidase subunit 4) as well as myoglobin mRNA are significantly up-regulated when training is carried out with an extra hypoxia stress. This is broadly compatible with observed functional and/or structural observations after training augmented by hypoxia (22; 31; 32; 36). Interestingly, Zoll et al (37) could demonstrate a significant correlation between the up-regulation of transcripts involved in pH regulation (CA3, carbonic anhydrase 3 and MCT-1, monocarboxylate transporter 1) and the time subjects could run at VO_2max. It is further suggestive that the observed increase in transcripts of factors mitigating oxidative stress (MnSOD, manganese superoxide dismutase and Cu/ZnSOD cytoplasmic copper/zinc dismutase) is related to the capacity of intermittent hypoxia training to perform respiratory function under low oxygen tension.

The observation of a complex and specific molecular response to the addition of a hypoxia stress to an endurance training protocol is intriguing and promising. We suggest that other tissues challenged by a training intervention such as the heart, the brain, the liver and the kidney may also react specifically to hypoxia. These changes remain to be determined but may be an important part of a hypoxia-modified global training response. It is further evident that not all changes that are invoked by hypoxia are necessarily beneficial for all athletes under all circumstances. Hypoxia favors glucose metabolism; this may be detrimental for athletes that could profit from developing their potential for fat oxidation (35).

TIME COURSE OF TRANSCRIPTOME CHANGES

In the previous paragraphs we have looked at the specific signature that a training intervention in hypoxia leaves on the steady state expression levels of performance relevant muscle genes. We now look at the way specific transcript levels change over

time after a single bout of exercise. The rationale here is that we expect transcript levels to change in a typical fashion over a 24 hour period between exercise bouts in response to the specifics of the stress that the muscle tissue was exposed to during exercise. We have looked at the time course of the muscle transcriptome changes using a custom made microarray containing 229 transcripts of interest for muscle plasticity (29). We took biopsies of untrained subjects before exercise as well as 1, 8 and 24 hours after a 30 min near exhaustive exercise bout on a bicycle ergometer. From 112 detected transcripts we found 23 transcripts to be significantly up-regulated while 3 were significantly down-regulated. The overall response of the transcriptome reveals a pronounced and significant drop of transcript levels at 1 hour post-exercise, a significant up-regulation over pre-test values at 8 hours post-exercise and a drop towards pre-test values at 24 hours post-exercise (Fig. 2; (29). After the initial exercise bout, subjects were endurance trained for 6 weeks (30 min/day; 5 days/week) before being subjected to a second exercise challenge and biopsy procedure, whereby the exercise load was adjusted to the training induced improvement in power output. Overall we found pre-test (steady state) concentrations of transcripts to be significantly increased. In the trained state the response to a single similar exercise challenge was massively attenuated. Instead of 20 transcripts being significantly up-regulated 8 hours post-exercise in untrained condition, only 2 were significantly up at 8 hours (29). More recently we have studied the time course of the transcriptome response over 24 hours following a mild bout of 15 min of eccentric work in previously untrained subjects (18) Since it has been documented that this type of exercise can lead to a considerable gain in muscle strength and in muscle fiber cross-sectional area, we expected to find an up-regulation of transcripts relevant for muscle growth. This was not the case. As indicated in Fig. 2, we saw an initial drop in transcript levels similar to that seen after concentric exercise. However, there was no evident immediate up-regulation thereafter and the general transcript level took 24 hours to return to close to pre-test values (Fig 2). With both training interventions the stimulus provoked an early down-regulation of transcription. This seems to be a normal reaction of tissue that has a high demand of energy and shuts down dispensable cellular reactions that use energy. For translational events it is quite well established that AMP-kinase, a sensor of intracellular energy levels is activated and inhibits translational events (12).

Taken together, the data from time course studies indicate that specific training protocols not only provoke specific changes of the transcriptional profile – but that these changes follow a discrete time course which is found to be different for different training interventions. Transcriptional profiling in a time series may therefore be a tool to optimize the timing of training interventions. In the case of eccentric exercise the current data suggests that the muscle takes more than 24 hours to recover and react to a preceding exercise bout.

CONCLUSIONS

Over the last ten years, molecular tools have started to provide insight into the expressional changes underlying muscle adaptational phenomena. The initial course of signaling events and their complex interactions have remained elusive; however the ensuing transcriptional response of muscle tissue starts to emerge and is seen to be specific in term of its character and of its time course. Exercise scientists using gene expressional screens on muscle tissue demonstrate a very detailed picture of the muscle tissue response. This will eventually enable them to tailor training regimes much more specifically in view of desired functional goals and test predicted functional outcomes with more selective and specific tests than those that are currently in use. This road seems particularly promising when exercise is used in clinical settings.

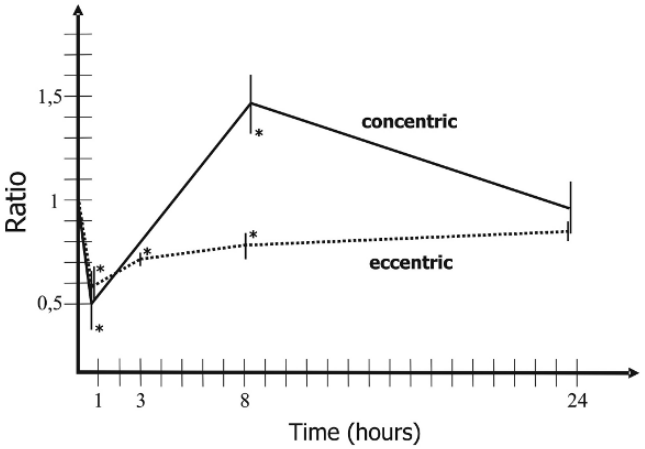

Figure 2. Time course of changes of the muscle transcriptome after a single bout of concentric or eccentric exercise. Exercise was carried out by previously untrained subjects at an intensity and duration typical on an initial training bout in the respective training proto col. Values for concentric exercise represent mean of 112 detected transcripts, values for eccentric exercise represent mean of 147 detected transcripts. The custom made microarray contained 220 transcripts. (Asterix indicates significantly different to pre-exercise value18; 29).

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Chapter 20

THE LIMITS OF HUMAN ENDURANCE: WHAT IS THE GREATEST ENDURANCE PERFORMANCE OF ALL TIME? WHICH FACTORS REGULATE PERFORMANCE AT EXTREME ALTITUDE?

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Abstract: Humans evolved as an athletic species able to run in the midday heat, to throw with exquisite accuracy and to strike powerfully despite relatively weak upper arms compared to those of the great apes. The true extent to which humans could run long distances was first tested in a unique series of 6-day foot races contested between 1874 and 1888 by professional athletes from England and the United States. These athletes typically would have expended approximately 60 000kcal (24.12MJ) of energy during these races. The discovery of the bicycle soon caused the replacement of these races by 6-day cycling races which, in turn, led to the modern day Tour de France, the cycling race across America (RaAM) and two running races across the width of the United States in 1928 and 1929. The total energy expenditures during these different events can be estimated at approximately 168 000, 180 000 and 340 000kcal respectively.

But, in terms of the total energy expenditure, all these performances pale somewhat when compared to that of Robert Falcon Scott's Polar party during the 1911/12 British Antarctic Expedition. For most of 159 consecutive days, Scott's team man-hauled for 10 hours a day to the South Pole and back covering a distance of 2 500km. Their predicted total energy expenditure per individual would have been about 1 million kcal, making theirs, by some margin, the greatest sustained endurance athletic performance of all time. Interestingly, the dogs that provided the pulling power for Norwegian Roald Amundsen's team that was the first to reach the South Pole, 35 days before Scott's party, would have expended about 500 000kcal in their 97 day trip, making theirs the greatest animal "sporting" performance on record. By contrast, mountain climbers expend only approximately 4 000kcal/day when climbing at extreme altitudes (above 4 000m). This relatively low rate of energy expenditure results from the low exercise intensities that can be sustained at extreme altitude. Here I argue that this slow rate of energy expenditure is caused, not by either myocardial or skeletal muscle hypoxia as is usually argued, but is more likely the result of a process integrated

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centrally in the brain, the function of which is to protect the body from harm. At extreme altitude the organ at greatest risk is the brain which must be protected from the catastrophic consequences of profound hypoxia. A key feature of this control is that it acts "in anticipation" specifically to insure that a catastrophic biological failure does not occur. The evidence for this interpretation is presented.

Key Words: energy expenditure, heart, central governor, evolution, hunting

INTRODUCTION

A critical determinant of the direction of human evolution occurred when our ancestors began to hunt antelope during day time heat on the hot African savannah. Exploiting this evolutionary niche, humans developed remarkable abilities as an athletic species able to run long distance, to throw with exquisite accuracy and to hit with power despite relatively weak arms. This in turn determined the way we look and perhaps even our mental capacity for persistent perseverance in pursuit of what we find appealing. The evidence underlying this hypothesis includes the following:

Humans have an unmatched capacity to sweat

Relatively few mammals other than humans, horses, donkeys and camels have a well developed sweating capacity. Thus some very large human athletes have the capacity to sweat at rates as high as 2-3 litres per hour during exercise (12, 16), enough to distribute as much as 1 500 kcal of heat to the environment every hour. This is enough to lose all the heat produced by a 70 kg athlete running at a speed of 24km/hr in moderate environmental conditions. Since no human can run this fast for more than a few minutes, this calculation indicates that humans have a maximal capacity to lose heat that exceeds by far their requirement under most normal conditions. This is surprising since it is usually held that, in the interests of biological economy, the human physiology is designed to have "just enough but not too much" capacity (10).

University of Vermont biologist, Bernd Heinrich has interpreted this accordingly: "The fact that we, as savanna-adapted animals have such hypertrophied sweating responses implies that, if we are naturally so profligate with water, it can only be because of some very big advantage. The most likely advantage was that it permitted us to perform prolonged exercise in the heat. We don't need a sweating response to outrun predators, because that requires relatively short, fast sprinting, where accumulating a heat load is like a lactic acid load, acceptable. What we do need sweating for, is to sustain running in the heat of the day – the time when most predators retire into the shade" (21) (p.174). Surviving hunter-gatherers, like the !Kung San (Bushmen) of Southern Africa have been filmed whilst running for 4-6 hours in temperatures of 40-46°C (11), until the body temperatures of their non-sweating prey, typically large African antelope like the Kudu and Eland, exceeded perhaps 42-45°C, causing motor paralysis (as the result of a failure of cortical motor drive to their exercising limbs - sometimes called central fatigue (14)). Thus the inability of otherwise too swift African antelope to sweat and so to lose heat at high rates when running for prolonged periods in midday heat is the defining biological weakness that our early human ancestors learned to exploit.

The exploitation of this evolutionary niche may also have developed other human characteristics. Mental and physical perseverance, for example, since no other predator shows such physical persistence when hunting. Then there was always the necessity to pace the hunt so that a measured effort could be maintained for the duration of the hunt without the frivolous wastage of precious energy that does not contribute to the end goal. The hunt must also be completed with functional reserve since the spoils of the capture must still be returned to the family home which was likely many kilometers distant. Finally these early hunters were the prototypical scientists since they were able to develop hypotheses and draw conclusions of the basis of information gleaned from the animal tracks left in the sand (27).

Thus any understanding of the physiology of modern humans must include the evidence that humans evolved to run in the heat at paces we could sustain for prolonged periods without the development of total exhaustion. Thus two crucial features of the physiology programmed into our genes by our evolutionary past are likely to be (i) the capacity to pace ourselves during exercise so that (ii) we complete each exercise bout with functional reserve and without the development of total physical exhaustion.

Yet, surprisingly, for the past century, exercise physiologists have attempted to understand our physiology, not by studying these two phenomena but by assuming that humans reach a state of complete physical exhaustion during exercise. But evolution probably adapted us specifically to insure that a state of complete fatigue does not ever occur in exercising humans.

Humans have developed an anatomical structure designed for running

Bramble and Lieberman (5) have identified 20 anatomical features of the human musculo-skeletal system which, they argue, are specific adaptations for sustained walking/running for prolonged periods (hours to days). Since humans are also the only mammals able to throw with exquisite accuracy and to strike powerfully using the torso and upper body, despite relatively weak arms (compared to the other apes), it is probable that these upper limb adaptations also evolved to improve our success as hunters. An obvious explanation would be the need to kill the exhausted prey either by striking it with a blunt instrument (in that period before human hunters had access to metal spears) or with a thrown object.

Humans have great capacity for endurance (36)

The peak running speed of the world's best human sprinters (10m/s) is far inferior to that of lions (~30m/s), cheetahs (~35m/s) and Thoroughbred race horses (~19m/s). But elite human marathoners can sustain running speeds of 5.6m/s for more than two hours equivalent to speeds of about 5.1m/s of the migratory African antelope, the wildebeest, and of 5.8m/s of the United States postal horses that carried the mail westwards before the coming of the railroad.

But no free living mammal covers greater distances than do elite Kenyan distance runners who may run as much as 30km daily (31).

It is these biological abilities that have allowed humans to perform remarkable ath-

letic performances. The aim of this paper is to establish which was the most enduring human athletic performance of all time. This will be defined as the athletic performance that required the greatest total energy expenditure.

Since the topic must also be relevant to the subject of human exercise in hypoxia, special attention will also be paid to the factors that might limit or regulate human exercise performance at extreme altitudes.

THE SIX DAY PEDESTRIAN RACES (1874- 1888)

In 1861 the American Edward Payson Weston walked 713km from Boston to Washington, DC to attend the inauguration of President Abraham Lincoln (31). He conceived the goal of walking 800km in 6 days (144hrs) which he achieved at his third attempt in December 1874 in Newark, New Jersey. This instigated a remarkable 14 year era of Anglo-American rivalry to determine which nation could produce the world's best (Six-Day) pedestrian. During this period the world record distance increased to a remarkable 1 004.2km established by Englishman, George Littlewood in 1888. Shortly after his remarkable achievement, pedestrianism "died" as a result of the development of the bicycle. Six day pedestrian races were soon replaced by equivalent Six Day Cycling races.

An estimate of the total energy expenditure of these pedestrians is provided by studies of the modern Greek pedestrian, Yiannis Kouros who, in March 1987, completed the 1060km Sydney to Melbourne race in 10 hours less than 6 days, equivalent to a final distance of ~1 127km for a full 144hr race (7.8km/h). Physiological studies (46) suggest that his total energy expenditure in a typical 6 day race was about 55 970kcal (average = 9 328kcal/day) with a peak of 15 367kcal on the first day. His average running speeds varied from 11.7km/h on the first day to 6.2km/h on the final day.

CYCLING - THE ORIGINAL 1903 SIX DAY TOUR DE FRANCE CYCLISTE LEADING TO THE MODERN TOURS DE FRANCE

Inspired by the popularity of the Six Day cycling races that superseded the Pedestrian races, in 1902 L'Auto-Velo, Geo Lefevre and Henri Desgrange, employees of the French sports daily, conceived the idea of introducing a six day cycling race around the perimeter of France, traveling from Paris to Lyon on the first day, and to Marseilles, Toulouse, Bordeaux and Nantes on the following four days, before finishing in Paris on the sixth day. They christened the race, the Tour de France Cycliste (64) (p.21-48).

The first race of 2 428km was won by Maurice Garin in 94 hours and 33 minutes for a daily average of 405km cycled in 15 hours 45 minutes at an average cycling speed of 26km/h. Since the average energy expenditure when cycling on the flat at 25km/h is about 400kcal/h but rises to 2 000kcal/h when climbing a 5% gradient at the same speed (24), the minimum total energy expenditure, assuming an absolutely flat course would have been about 38 000kcal and the average daily energy expenditure about

6 300kcal.

In keeping with the spirit of the times, the difficulty of the race increased progressively in the 1920's as the race distance increased, reaching a peak distance of 5 700km in the 1926 race. In addition, mountain stages in the Pyrenees and Alps were included. Thereafter the race distance has progressively reduced to the modern distance of about 3500km. However the average speed of the race is now in excess of 40km/hr. Since the energy cost of cycling is an exponential function of speed (24), this reflects an approximately three-fold increase in the ***rate*** of energy expenditure by modern cyclists compared to the early pioneers.

In 1988 Saris and colleagues (47) from the University of Maastricht showed that the average daily energy expenditure of cyclists in the Tour de France was about 8 000kcal per day so that the total energy expenditure for the complete 21 stages of the race would have been about 168 000kcal.

Interestingly the total "effort" expended in professional cycling races is to some extent independent of the total distance of the race. Thus from heart rate measurements on the same 7 cyclists competing in the Tour de France and the Tour of Spain, Lucia et al (28) showed that the total heart rate "load" was the same in these races even though the cyclists took about 500 minutes (10%) longer to complete the Tour de France. This suggests that the brain paces the body not just in a single exercise bout but also in events lasting many weeks, choosing to expended similar amounts of energy during competitive performances of similar importance (to the athlete) but of different durations.

Another interesting aspect of the Tour de France is the nature of the drugs that cyclists use to improve or sustain their performances. Jacques Anquetil who won the race five times in the 1950's and 60's admitted that he used a combination of four drugs – the painkillers morphine and palfium which he injected directly into his tired leg muscles; the amphetamines tonedron and pervetin; a "lung opener" solucampre and a sleeping tablet, gardenal. The important point is that none of these drugs acts to limit "peripheral fatigue" since especially morphine and the amphetamines act centrally in the brain. One might suggest that the nature of the fatigue that Tour de France cyclists wish to eliminate by drug use must be generated by the brain (as part of a complex regulatory system (41)).

Similarly, the average racing speed in the Tour de France has increased in the past 17 years (Figure 1), perhaps related to the introduction of Erythropoietin (EPO) in the early 1990's. But it is not immediately clear how EPO can improve submaximal exercise performance in prolonged events like the Tour de France, if the principal effect of EPO is to increase the red cell mass and the hemoglobin (Hb) concentration of the blood.

For if submaximal exercise performance were truly limited by an inadequate Hb (and oxygen) flow to muscle, then the cardiac output would have to be maximal even during submaximal exercise. But it is not. This logic suggests that EPO improves submaximal endurance performance by mechanisms not related simply to increased Hb and oxygen delivery to the exercising muscles.

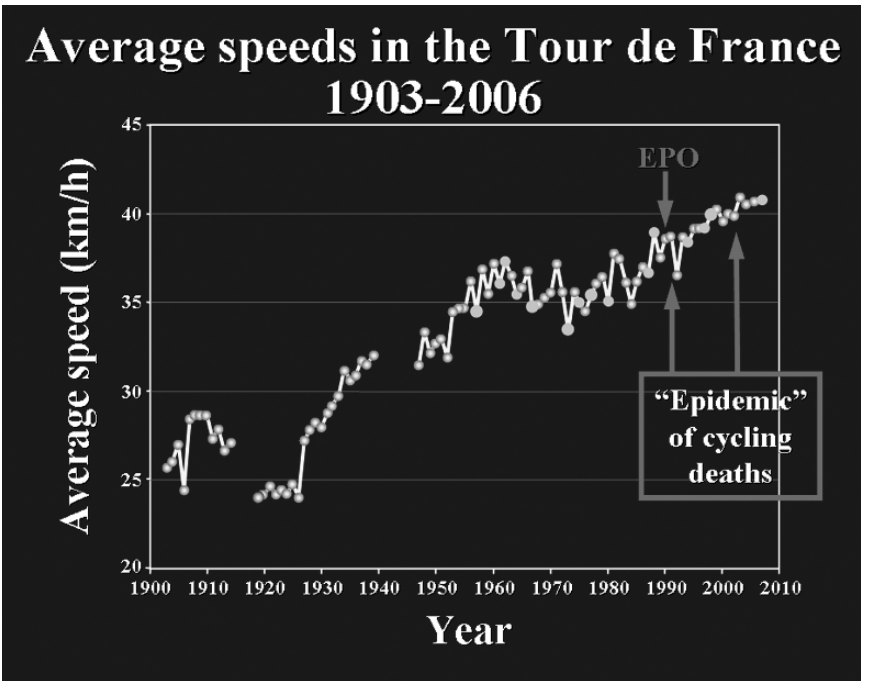

Figure 1. The average racing speeds in the Tour de France. Note that average speeds were relatively stable between 1960 and 1990 but increased progressively thereafter. It is believed that the drug erythropoietin (EPO) was first introduced into cycling in the late 1980's (arrowed) (35). An unusually high rate of deaths amongst professional cyclists occurred in 1989 and again in 2003 (arrowed). It is suspected that these deaths may have resulted from the unrestricted use of novel drugs like EPO by persons naïve to the associated risks (35).

THE C.C. ("CASH AND CARRY") PYLE UNITED STATES TRANSCONTINENTAL RACES OF 1928 AND 1929

The success of the Tour de France in turn inspired a North American entrepreneur, C.C. Pyle, to attempt a similar event in the United States of America. But he chose a running race across the breadth of North America, from Los Angeles to New York, a distance of 4 960km. The first race began on Sunday March 4th 1928 in Los Angeles and was completed in 84 stages (average stage distance of 59km) (3) before finishing in New York. The following year the direction was reversed and the number of stages reduced to 78 (average stage distance of 64km). The first race was won by A. Payne who covered the distance in 573 hours 4 minutes and 34 seconds at an average pace of 8.7km/hr; the return race was won by J.Salo who had finished second in the first race. His time was 525 hours 57 minutes and 20 seconds at an average pace of 9.5km/hr. The doctors who studied the athletes before and after the race concluded that: "the comparatively normal human body provided with adequate food and rest, may acquire during prolonged exercise, unusual capacity for work apparently without serious untoward effect" (17). This despite the fact that most of the athletes were completely unprepared

for the event and had little knowledge of their nutritional needs or fluid requirements during the race.

A reasonable calculation suggests that the minimum hourly energy expenditure of a 65 kg athlete running on the flat at a pace of between 8.7-9.5km/hr would likely be about 600kcal per hour or about 4 300kcal per day (31). Thus the total energy expenditure during the race would have been about 340 000kcal or about seven times the energy expenditure during the Six Day Pedestrian races.

More recently, a study of a single athlete completing the cycling Race Across AMerica (RAAM) found that his daily energy expenditure was about 18 000kcal for a total energy expenditure of 179 650 for the 9 days and 16 hours that he required to complete the crossing (26). These findings are very similar to the energy expenditure during the Tour de France and about half the expenditure during the US Transcontinental Bunion Derbies of 1928 and 1929.

THE EXPLORERS OF THE SOUTH POLE DURING THE "HEROIC" ERA

The discovery of the Great Southern Continent in the middle of the 19th Century inspired an international race to claim its ownership. The British were the first nation to commit substantial resources to the quest. They supported a series of expeditions in the first two decades of the twentieth century, the ostensible goal of which was to be the first nation to reach the South Pole. In the end this nationalist goal was eclipsed by the performance of the Norwegian team led by Roald Amundsen, who reached the South Pole on December 14th 1911, 35 days before the British team of Robert Falcon Scott. The British were defeated because they chose to man-haul their provisions to the Pole and back whereas the Norwegians used dogs to accomplish the task more efficiently (23).

Thus Scott's party, relying mainly on "heroic" manhauling, covered the 1 400km to the South Pole in 86 days. They would man-haul a further 1 082km before frostbite to Scott's legs caused by the unseasonably cold weather (50), doomed him to die. For the low temperatures increased the frictional resistance of the ice until the effort became similar to pulling the sled through sand. Under these conditions, Scott's two healthy companions, Wilson and Bowers, would not have been able to drag Scott the final 300km to safety. Instead they chose to wait with Scott until he died. But Scott was the last to die. This conclusion is based on new information, only recently appreciated (50). That Scott's two surviving companions chose to die with him rather than to make their own dash for life forces a re-consideration of Scott's character and influence despite clear evidence for his many failings (23).

By man-hauling essentially for 159 consecutive days, the last 60 of which were in extreme cold, for a total of 2 500km, the feat of Scott and his colleagues is, in my opinion, the greatest human performances of sustained physical endurance of all time.

The total energy cost of their effort can be reasonably estimated from data collected on other man-hauling expeditions. In the Southern summer of 1985/6, Englishmen

Gareth Wood, Roger Swan and Roger Mear retraced the first half of Scott's 1911-12 trip by man-hauling all their supplies, unsupported, the 1410km from McMurdo Sound to the South Pole in 70 days. Doubly labeled water studies found that their daily energy expenditure was between 6 000 and 6 900kcal (53) for a total energy expenditure of about 455 000kcal. These data for daily energy expenditure under these conditions are very similar to those measured on Dr. Michael Stroud and Sir Rannulph Fiennes during a 48day expedition to the North Pole (54).

Stroud (52) concluded that Scott's team might have sustained a daily energy expenditure of between 5 900 and 6 900kcal for 159 days for a total energy expenditure of perhaps ~1 000 000kcal or nearly twice the expenditure of The Footsteps of Scott expedition. Stroud also estimates that the daily food intake of Scott's polar party varied from about 4 500kcal on the southern trip to about 3 800kcal on the return trip, for a daily energy deficit of about 1 500-2 400kcal. This leads to the conclusion that Scott died from starvation rather than from "peripheral fatigue". Indeed, of the five members of Scott's Polar Party, two (Scott from incapacitating frost bite; Evans from a cerebrovascular accident probably related to scurvy) died from "natural" causes whereas the remaining three (Oates, Wilson and Bowers) all chose to end their lives. This suggests again that humans function with "reserve" even until the moment that they die.

The most exceptional modern Polar performances is that of Sir Ranulph Fiennes and Dr Michael Stroud (52, 55, 56) who man-hauled their own supplies for 95 consecutive days covering 2 300km in their attempt to cross the Antarctic Continent from the Weddell to Ross Sea, as Shackleton had planned for his 1914/15 expedition. Like Scott's parties, Fiennes and Stroud man-hauled for 10 hours most days. Despite eating 5 070kcal per day, both explorers lost more than 20kg of body mass. Whilst there was some discrepancy in the calculated energy expenditures measured with doubly labeled water, values were generally in excess of 5 500kcal/day and apparently reached as high as 10 000kcal/day during one 10 day period (56). Thus the total energy expenditures of Fiennes and Stroud may have been at least 570 000kcal, a record for a self-supported expedition.

When Stroud and Fiennes terminated their expedition they were close to death, yet still they maintained just enough reserve to survive their rescue.

Interestingly, the total daily energy cost incurred by dogs pulling sleds is equivalent to that of humans. Thus sled dogs require a daily intake of 5000 kcal if they are to maintain condition on sledding trips (43). Accordingly, the amount of energy expended by the 11 surviving dogs that powered Roald Amundsen's successful 97 day round trip to the South Pole and back in 1911, would have expended about 500 000kcal during the expedition. This is perhaps the greatest recorded animal "sporting" performance. Note that their total energy expenditure was about one-half that of Scott's Polar party, in line with the conclusion that dogs are about twice as efficient as humans at hauling sleds since they travel twice as far on the same daily energy intake (43).

MOUNTAINEERING EXPEDITIONS TO HIGH ALTITUDE

Energy expenditure at high altitude is limited by the inability of humans to survive for prolonged periods at altitudes above about 6 000m and the incapacity to perform work at high rates at such altitudes. Thus measurements of two climbers on Mount Everest showed that their energy expenditure during 12 days of climbing at altitudes of from 5 000 to 8 000m for up to three and a half hours a day varied from 2 900 to 4 000kcal per day (63). These quite low rates of energy expenditure occur because of the inability to exercise at a high intensity at altitude and the short durations of daily exercise undertaken by climbers at these extreme altitudes.

Indeed, of some interest is the biological explanation for the impaired exercise performance at high altitude. There are two opposing theories: The first holds that the function of the exercising muscles (and the heart) becomes increasingly impaired as a result of the progressively more severe hypoxia that develops at higher altitudes. This model of peripheral regulation or "peripheral fatigue" has been termed the cardiovascular/anaerobic model (40). When taken to its logical conclusion, this model predicts that the heart determines, indeed regulates the function of the exercising muscles by deciding how much blood it will choose to deliver to the muscles during exercise so that they may perform at a level that the heart considers appropriate. This might also be described as an "oxygen push" model in which the exercise performance is determined by how much oxygen is "pushed" to the muscles (38).

The contrasting model theorizes that the human physiology is regulated, not limited, during exercise specifically to insure that organ damage does not occur. This model has been termed the central governor model (41), in honor of Nobel Laureate Professor AV Hill on whose work the opposing "oxygen push" model is paradoxically based (34). Less frequently acknowledged is Hill's appreciation that his model predicts that the heart will always be the first organ to fail (as a result of ischemia) if exercise performance is indeed limited by the heart's failure to increase the cardiac output (34). Thus Hill proposed the existence of a "governor" in the brain or heart to prevent irreversible damage to the heart when this inevitable ischemia developed: "We suggest that… either in the heart muscle itself or in the nervous system, there is some mechanism (a "governor") which causes a slowing of the circulation as soon as a serious degree of unsaturation occurs…" (22).

We have expanded this interpretation to propose that a regulator or governor in the brain would control the exercise performance, not by limiting heart or skeletal muscle function as a result of biological failure (fatigue), but by regulating the number of motor units that can be activated in the exercising skeletal muscles by the motor cortex under different conditions of exercise (51).

The published evidence that must be considered when choosing which of these two different models best explains the physiological basis for the exercise impairment in hypoxia, includes the following:

The respiratory muscle paradox

Already in 1988 Bigland-Ritchie and Vollestadt (4) recognized that one set of

muscles, the respiratory muscles, are clearly not affected by the hypoxia of extreme altitude since rates of ventilation are often higher in hypoxia than in normoxia. They therefore wondered how lactic acid, which according to the "oxygen push" model is the "poison" that limits exercise performance (40), can act exclusively on the exercising skeletal muscles and not on the respiratory muscles or even the heart. They wondered: "Why should hypoxia have such a profound effect on limb muscle performance, while work capacity of the respiratory muscles seemed unaffected when both muscle groups were performing similar types of dynamic exercise?" (4) (p 375). Rather they suggested: "…it is essential for survival that somehow respiratory muscles must avoid the extremes of fatigue experienced by limb muscles…. This could be achieved if CNS strategy involves some kind of reciprocal inhibition between the motor drive to limb muscles, with that from the respiratory system dominating. According to this scheme, the motor drive to limb muscles can achieve and retain maximal muscle activation, provided this does not increase metabolic demand above that which the respiratory muscles can deliver. However, if either the muscle mass is too large or the exercise sufficiently demanding, such that metabolism exceeds the capacity of the oxygen delivery system, a balance between them is restored by an automatic reduction of motor drive to limb muscles. In this case, the limb muscles can no longer be fully activated and central fatigue develops. …. Thus, fatigue developed under these conditions may have been caused more by a reduced motor drive than by peripheral factors (so that) … taken together, these observations support the concept that the motor drive to limb muscles is reduced when the metabolic demand of skeletal muscle exceeds that which the respiratory muscles can supply." (p 375).

This possibility that "a reduced motor drive" may explain the impaired exercise performance at altitude is discussed subsequently.

The lactate paradox

Since at least the 1930's, it has been known that peak blood lactate concentrations at exhaustion during "maximal exercise" fall exponentially with increasing altitude (Figure 2- left panel) so that peak concentrations measured during maximal exercise at the equivalent of the summit of Mount Everest are no higher than resting values (62). This is clearly paradoxical according to the model which holds that (a) muscles which are "anaerobic" or "hypoxic" produce lactic acid and that this production must be maximal during exercise in extreme hypoxia and (b) that lactic acid is the "poison" that causes (peripheral) muscle fatigue to develop. Indeed the conclusions from Operation Everest II were that:"… neither substrate availability nor metabolic product accumulation limited exercise capacity at extreme simulated altitude" (18) (p 2574). Nor did that study find any support for an hypothesis that "at the level of the muscle cell, extreme hypobaric hypoxia elicits adaptations directed towards maximizing oxidative function" (p 2454).

These findings are compatible with the interpretation that, during exercise in severe hypoxia, the skeletal muscles cells are actively protected from a catastrophic homeostatic failure caused by the development of profound cellular hypoxia. Few have sought to determine the nature of that control.

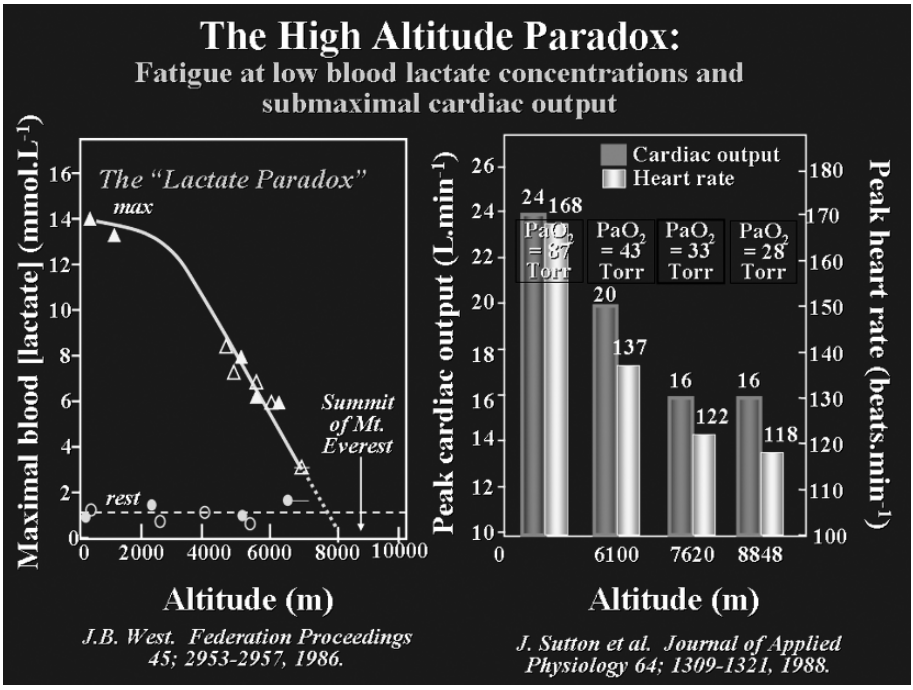

Figure 2. The left panel shows that blood lactate concentrations during maximal exercise fall with increasing altitude. This is paradoxical according to the model which holds that skeletal muscle anaerobiosis, worsened at altitude, causes the termination of exercise as the result of a profound skeletal muscle lactic acidosis. The right panel shows that cardiac output and heart rate also fall during maximal exercise at increasing altitude. This too is paradoxical since according to the "oxygen push" model of exercise performance, cardiac output should increase at increasing altitude in order to maximize blood and oxygen delivery to the exercising muscles.

The cardiac output paradox

Less well recognized is the associated cardiac output paradox in which exercise at extreme altitude also terminates at low cardiac outputs and low heart rates (57) (Figure 2 – right panel). This is paradoxical because, according to the "oxygen push" model of exercise performance, the assumption must be that the cardiac output will increase progressively as the oxygen content of the blood falls with the increasingly severe hypoxia that develops at higher altitudes. For this is the only manner by which the heart can maximize its provision of oxygen to the muscles and hence to optimize the exercise performance (which, according to the "oxygen push" model, is controlled by the heart).

Instead leg blood flow is actually reduced during exercise after altitude acclimatization (2) (since the arterial oxygen content is increased). This indicates that the blood flow responds to the oxygen demands of the tissues. The blood flow (cardiac output) does not set that demand (as is required by the "oxygen-push" model).

Left ventricular function is not impaired during exercise at extreme altitude

The presence of left ventricular dysfunction can be detected by a change in the relationship between the left ventricular end diastolic volume and some measure of heart function, usually the stroke volume. This relationship is not altered during maximal exercise at extreme altitude (44) confirming that the function of the heart is maintained at extreme altitude even in the face of a large increase in pulmonary artery pressure (19). Since myocardial hypoxia, ischemia or anaerobiosis rapidly impairs left ventricular function, the presence of normal heart function in hypoxia must indicate that myocardial oxygenation is normal even during maximal exercise on the summit of Mount Everest, as also confirmed by the absence of diagnostic electrocardiographic changes during maximal exercise (29). These facts are not always acknowledged (38, 60).

An elevated hematocrit produced by either EPO administration or blood transfusion or prolonged altitude acclimatization does not improve performance at altitudes above about 4000m

Already in 1996, Young et al (65) showed that the maximum oxygen consumption (VO_{2max}) measured at 4300m altitude is not influenced by an erythrocyte infusion that increased the Hb concentration by 10%. They concluded that: "At high altitude, VO_{2max} may be limited by other factors (than cardiac output and arterial oxygen content)" (p 257).

Similarly the increase in Hb content and red cell mass that occurs with acclimatization fails to improve maximal exercise performance at an altitude equivalent to 5260m (7). But switching the nature of the inhaled air from a hypoxic to a hyperoxic mixture at the point of exhaustion during maximal exercise at simulated or real altitude instantly reverses the symptoms of fatigue and allows the exercise to continue (6, 25).

The work of Robach et al. (45) presented at this conference shows that whereas EPO administration reduces the extent to which exercise performance is impaired at altitudes up to 3500 m; this effect is lost at an altitude of 4500m. This indicates that increasing the oxygen content of blood alleviates the detrimental effects of hypoxia only until some critical degree of hypoxia is reached.

How might these unexpected findings be explained?

Patterns of skeletal muscle recruitment are altered at altitude

Some years ago we completed an experiment to assess whether or not a prospective Mount Everest climber could expect to reach the summit of Mount Everest without the use of supplemental oxygen. Accordingly, we exposed him to short bouts (3 minutes) of exercise in a sealed chamber in which the inspired oxygen fraction (FiO_2) could be altered. Over a period of 3 hours we lowered the FiO_2 from 0.21 (equivalent to sea level) to 0.07 (equivalent to the summit of Mount Everest) and monitored the subject's cardiac output (by right heart catheterization), ventilation and arterial oxygen (PaO_2) and carbon dioxide ($PaCO_2$) partial pressures. Figure 3 shows that, with increasing altitude, the PaO_2 and $PaCO_2$ fell progressively reaching very low levels at an FiO_2 equivalent to the summit of Mount Everest. At this point the climber lost consciousness, im-

mediately after completing his final exercise bout. His collapse was characterized by a precipitous fall in arterial oxygen saturation immediately on exercise termination.

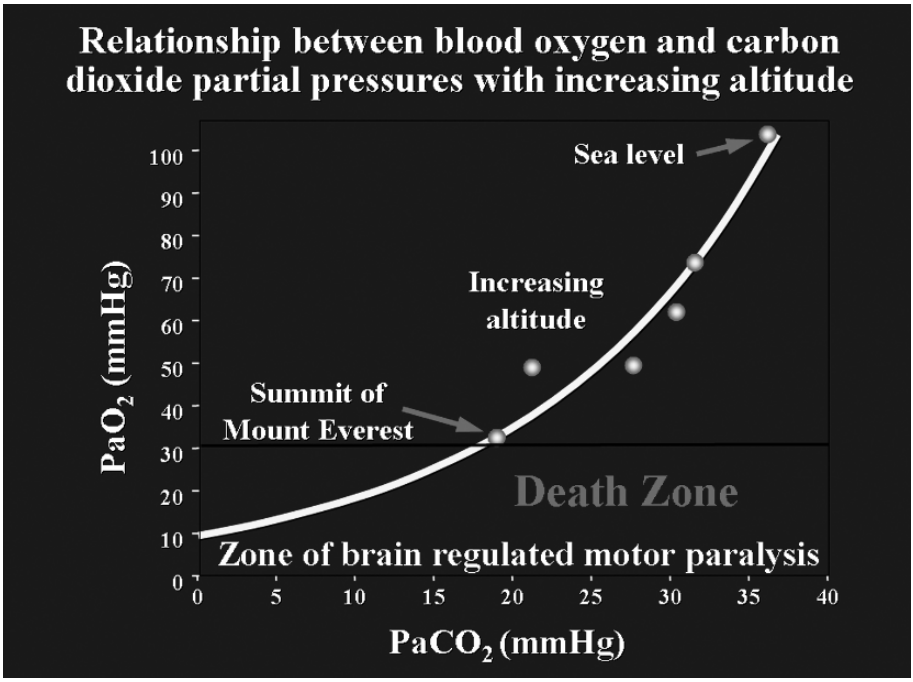

Figure 3. The arterial partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) both fall with increasing altitude. One possibility is that when these fall below some critical value, the brain reduces the extent to which it will recruit motor units in the lower limbs. In the Everest Death Zone when the PaO_2 is ~30 Torr, it is proposed that a state of complete motor paralysis is produced (as evidenced by the motor paralysis that strikes some Everest climbers at very high altitudes). At higher PaO_2 there is evidence for a progressive reduction in the extent of skeletal muscle recruitment during exercise (Figure 4). It remains unclear what variable is being "protected" when the PaO_2 is higher than that at which cerebral hypoxia develops, risking brain damage. (Data from a single experiment on mountaineer Alex Harris, Cape Town, 21st November 2002).

Our conclusion is that a functional muscular paralysis occurs at very low PaO_2 and $PaCO_2$ probably as a result of profound cerebral hypoxia and perhaps regulated by a combination of low PaO_2 and $PaCO_2$. Indeed the brain would "know" at what altitude it is by monitoring these two variables (Figure 3). If correct, this would explain why EPO, which does not influence either the PaO_2 or the $PaCO_2$, would not improve exercise performance at those levels of advanced hypoxia at which the PaO_2 and $PaCO_2$ (may) become the sole variables regulating the exercise performance. At lower levels of hypoxia at which neither of these variables is the exclusive regulator of the exercise performance, EPO would indeed have an effect (if it alters the variables that regulate maximal exercise performance at those altitudes). In which case the variables regulating the exercise performance could be either the red cell mass or the Hb concentration

or even perhaps some other unrecognized factor(s) that are influenced by EPO. It is known for example that EPO has some effects in the brain and its use improves the well-being of patients with severe anaemia, well before their Hb content of red cell mass is normalized.

This interpretation that alterations in skeletal muscle recruitment by the brain may explain the changes that occur in exercise performance at altitude is based on the following information:

When Reinhold Messner reached the summit of Mount Everest on May 8th 1978, he described his experience thus: "We can no longer keep on our feet to rest … Every 10 - 15 steps we collapse into the snow to rest, then crawl on" (30). Were Messner's symptoms due solely to the development of lactic acidosis causing leg muscle fatigue (according to the "oxygen push" model), he would still be able to walk and would not need to crawl to the summit of Mount Everest. Rather crawling indicates that the patterns of motor recruitment of the muscles of the leg and the trunk by the brain have been fundamentally altered. Crawling to the summit of Mount Everest therefore represents an altered pattern of muscle recruitment that is determined by the brain and which must have some biological value.

Indeed, the evidence that the recruitment of skeletal muscle is altered by hypoxia, as perhaps first proposed by Bigland-Ritchie and Vollestadt (4) is very well established in the scientific literature although it seems to have been overlooked.

For example, a study (15) performed as part of Operation Everest II and which is seldom remembered, concluded that "central motor drive becomes more precarious at altitude and is associated with increased muscle fatigue at low excitation frequencies" (p 1167). Then in 1994, Kayser et al. (25) established that the electromyographic (EMG) activity in the lower limb muscles was reduced during exercise at an altitude of ~5 000m. This is usually interpreted as evidence that the extent of motor unit recruitment has fallen. Accordingly these authors proposed that: "These results suggest that during chronic hypobaric hypoxia, the central nervous system may play a primary role in limiting exhaustive exercise and maximum accumulating of lactate in blood**".** More recently Fulco et al. (13) have shown that central recruitment during repetitive maximal voluntary contractions fell more in hypoxia than in normoxia further suggesting that hypoxia alters the brain's capacity maximally to recruit the exercising muscles.

Surprisingly, these finding have been integrated hardly at all into the understanding of the factors that limit exercise in hypoxia (38). This is perhaps a natural consequence of Operation Everest II which placed its major focus on the cardiovascular (9, 44, 57) and respiratory (61) adaptations to extreme altitude, generating the presumption that cardio-respiratory factors must determine maximal exercise performance also at extreme altitude.

Recently Amann et al. (1) have confirmed and extended these findings. By measuring lower limb EMG activity during self-paced 5km cycling time trials at different FiO_2, they showed that EMG activity was reduced progressively with increasing hypoxia as the FiO_2 fell from 1.0 to 0.28, to 0.21 to 0.15 (Figure 4). They also showed that (peripheral) skeletal muscle function in response to stimulation of the femoral nerve was reduced to the same extent after exercise regardless of the FiO_2. Thus any difference in exercise performance at different FiO_2 could not have been due to the development

of a greater "peripheral fatigue" with increasing hypoxia. Instead they postulated that, in response to increasing sensory feedback from the fatiguing peripheral muscles, a central brain mechanism reduces skeletal muscle recruitment, hence impairing performance. They argued that this mechanism would prevent the development of a "critical" level of peripheral fatigue.

Whilst this interpretation represents a major advance in the understanding of the control mechanisms regulating exercise performance in hypoxia and corresponds to our previous proposals (32-34, 38-41), yet it fails to explain all the findings of those authors. A slightly modified interpretation, that is also better able to explain other findings (42), would appear to be more plausible.

In the first place, these authors reported that EMG activity in the rectus femoris muscle was already different between the different FiO_2 conditions within the first 10% (500m) of the time trials. This difference then remained for the duration of exercise (Figure 3 in (1). Since the levels of "peripheral fatigue" were the same when measured at the end of exercise in all conditions, this finding effectively dissociates (equivalent) levels of "peripheral fatigue" from (different) levels of skeletal muscle recruitment showing that the one ("increasing peripheral fatigue") cannot cause the other (different levels of reduced central recruitment of the exercising muscles). Furthermore the fact that EMG activity had fallen so precipitously within the first 500m of the cycling time trial in the lowest FiO_2 of 0.15 indicates that this must be an "anticipatory" response to sensory feedback from something other than "peripheral fatigue", since such fatigue could not already have been maximally developed within the first 40-50 seconds of exercise. In view of the rapidity of this response, the most likely sensory information that could produce an essentially instantaneous effect would be a sudden fall in either the PaO_2 or $PaCO_2$ on exposure to the low FiO_2. The existence of just such an "anticipatory" response to sensory feedback during exercise in the heat is well established (58, 59).

Second, in all time trials except that at the lowest FiO_2, EMG activity and cycling power output increased during the final 1km (20%) of the time trial reaching the highest values at the finish of the time trials at FiO_2 of 1.0 and 0.28 (Figure 3 in (1)). This end spurt is a well recognized phenomenon; its critical relevance to the understanding of human exercise physiology has been described (37, 40). Since the levels of "peripheral fatigue" must have been greatest at the end of exercise, this finding further dissociates the development of (an increasing peripheral) skeletal muscle fatigue from (an increased) skeletal muscle recruitment during the end spurt. According to the model proposed by these authors, the end spurt should not have been possible since high levels of "peripheral fatigue" should have prevented any increase in muscle recruitment by the brain at the end of exercise. In contrast, if the end spurt is an "anticipatory" response that is activated by the brain (which alone has the knowledge that the finish is close), then this response could indeed over-ride any effects of "peripheral fatigue". Indeed the critical importance of the end-spurt is to show that the body always has a functional reserve even when the effort appears to be "maximal".

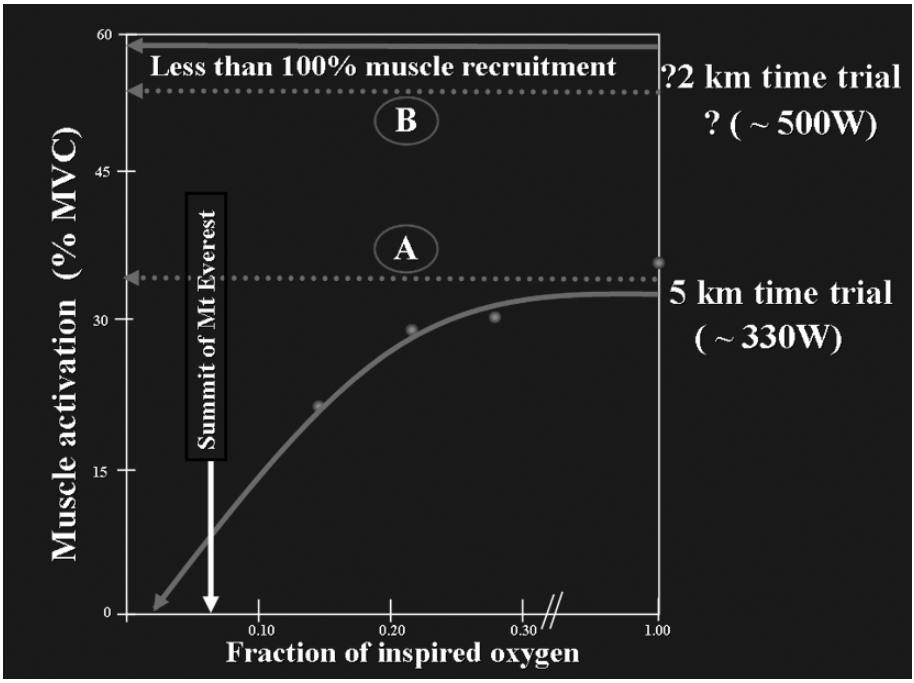

Figure 4. The study of Amann et al. (1) showed that the extent of skeletal muscle recruitment during a 5km cycling time trial falls with reducing FiO2. Projected to lower FiO2 these data would predict that a state of total muscular paralysis will occur at the FiO2 expected to be present near the summit of Mount Everest. Line A shows that when subjects were asked to perform a 5km cycling time trial, they cycled at an average power output of about 330W and recruited about 35% of the available motor units in their rectus femoris muscles (Line A). Since this value is less than 100% it indicates that performance in a 5km cycling time trial is regulated by the brain which determines the pace by choosing the number of motor units it will allow to be recruited in the exercising limbs. But if the subjects had been told they would be cycling for only 2km, they would achieve a higher average power output which would have to be the result of a greater skeletal muscle recruitment (Line B). But since this value is still less than 100%, even at this higher exercise intensity, the performance is still regulated principally by the brain (although it may be influenced to some extent by the development of "peripheral fatigue"). However the large recruitment reserve that exists even when exercise lasts for only a few minutes (Line B) indicates that the brain could off-set any effects of "peripheral fatigue" on performance simply by recruiting a greater number of motor units in the exercising limbs. That it chooses not to do this indicates that, whereas athletes, scientists and coaches may believe that the athlete's sole purpose is to travel "as fast as possible", the brain has a quite different agenda. Its function is presumably to protect athletes from killing themselves (41) especially at extreme altitude.

Third, levels of skeletal muscle recruitment were substantially submaximal throughout the exercise bout (Line A in Figure 4) so that only about 35% of the available leg musculature may have been activated during the exercise bout at this intensity. All

these findings can perhaps be better explained according to a slightly different model (58, 59)

Prior to the onset of exercise, the brain anticipates the number of motor units that it will allow to be recruited from the start of the exercise bout. This decision is based on prior experience and the expected duration of the exercise bout. Thus subjects in the study of Amann et al. (1) chose an average work rate of ~330W when told beforehand that they would be exercising for only 5km (in an FiO_2 of 1.0). As a result, subjects initially recruited only about 35% of the available motor units in the rectus femoris muscle (Line A in Figure 4). But if they had been told beforehand that they would be exercising for only 2km, they would likely have sustained a much higher average power output, say ~500W, which would have required the recruitment of many more motor units, say about ~55% of those available (Line B in Figure 4). The crucial point is that if less than 100% of the available muscle mass is recruited either during exercise or at the point of exhaustion, then the brain is regulating the exercise performance (40)

The additional important information provided by the study of Amann et al. (1) is that the extent of skeletal muscle recruitment during exercise at different FiO_2 was already different within the first 10% of the exercise bout and remained relatively constant thereafter until increasing during the end spurt at the higher FiO_2's. This indicates that (i) at the start of exercise the brain sets the level of skeletal muscle recruitment that, on the basis of past experience, it "knows" the body will be able to sustain for the expected exercise duration (compare lines A and B in Figure 4) (and which is also known before the exercise begins) but (ii) the extent of skeletal muscle recruitment is open to modification by any special circumstances arising during exercise, for example, the presence of an unexpectedly low FiO_2 (about which the brain had no prior knowledge in these studies).

Thus, on the basis of feedback from multiple organs in hypoxia, as already shown during exercise in the heat (58, 59), the brain rapidly resets the extent of muscle recruitment shortly after the onset of exercise. This clearly must have some biological value. We interpret this as another example of a control mechanism which functions to sustain homeostasis; to retain a reserve and to insure that exercise terminates before a catastrophic failure develops (32, 40). Thus the study of Amann et al. (1) clearly establishes that this mechanism also exists in hypoxia, presumably to prevent the development of cerebral hypoxia.

Finally the studies of Kayser et al. (25) and Calbet et al. (6) provide evidence that the PaO_2 is the most likely candidate as the sensed variable (Figure 3) influencing the level of skeletal muscle recruitment during exercise in hypoxia. For both those studies found that exercise performance improved essentially instantly when an hypoxic condition was reversed by increasing the FiO_2. Indeed Calbet et al concluded: "…the fact that it was possible to continue the incremental exercise test with re-oxygenation argues against a peripheral (muscular of metabolic) mechanism as the main cause of fatigue in severe acute hypoxia" (6) (p 300). In contrast increasing the arterial oxygen **content** by increasing the red cell mass either with a blood transfusion (65), with EPO administration (45) or with altitude acclimatization (7), produces no such effect.

Since it is the PaO_2 that determines cerebral function (whereas the arterial oxygen content is more likely the determinant of skeletal muscle oxygenation), this further

implicates the brain as the regulator of exercise performance in hypoxia.

SUMMARY

When judged by the amount of energy expended on a daily basis for sustained periods, the limits of human endurance would seem to have been set by a series of remarkable human performances by the early Polar explorers at the start of the last century. Under the most extreme conditions of cold, discomfort and semi-starvation, they sustained high daily rates of energy expenditure (~6 000kcal/day) for more than 100 days. These total energy expenditures are more than three to six fold greater than those sustained by the modern sporting heroes, for example the cyclists in the Tour de France. Their basis for their extraordinary performances can be more easily explained: Once they began, they could not afford the luxury of stopping. For as Shackleton who twice raced before the clutches of death has written: "Our food lies ahead, and death stalks us from behind" (49), (p.358).

The question may be asked: Under these conditions, what determines the limits of human endurance? Clearly in the Polar regions, starvation as a result of being unable to carry enough food and frost bite made more probable by the early onset of the deep winter already in March in the Antarctic (50), are the key factors that limit the extent to which humans can test their endurance. There are essentially only four months in which it is possible to walk on the Antarctic continent; Scott's Polar party tested the limits of that time window.

Apsley Cherry-Garrard, a member of Scott's final expedition, and who himself undertook a 105km walk in 35 days with Wilson and Bowers in the Antarctic midwinter of 1911 in temperatures of -50 to -60°C in search of the egg of an Emperor Penguin, wrote that: "It was the sensitive man, the men with nerves, with a background of education – 'good blood' – who went farthest, pulled hardest, stayed longest….. Other things being equal, the men with the greatest store of nervous energy came best through this expedition. Having more imagination, they have a worse time than their more phlegmatic companions; but they get things done. And when the worst came to the worst, their strength of mind triumphed over the their weakness of body. If you want a good polar traveler, get a man with too much muscle, with good physical tone, and let his mind be on wires – of steel. And if you can't get both, sacrifice physique and bank on will" (8).

To which can be added Sir Ernest Shackleton's five criteria for selecting the men for his Endurance expedition, perhaps the ultimate epic of Polar Exploration (48): Optimism, Patience, Physical Endurance, Idealism and Courage (20) (p.47). Only one of these five characteristics, physical endurance, was considered by Shackleton to be of the body; four are of the mind. But we now know that physical endurance is in fact, just a reflection of mental endurance.

For when the activity is prolonged, the mind not the body becomes the ultimate determinant of what can be achieved. The limits of our endurance lie deeply in the human brain, determined by our heredity and other personal factors yet to be uncovered. It is the mind that determines who chooses to start and who best stays the distance.

Similarly, we argue that exercise performance at extreme altitude is regulated by a complex intelligent system in the brain, responsive to the arterial PaO_2 and perhaps the $PaCO_2$, and the function of which is to protect the brain from severe arterial hypoxia. The mechanism of control is through a direct alteration in the extent of skeletal muscle recruitment. Since the extent of skeletal muscle recruitment determines the rate of skeletal muscle metabolism, oxygen consumption, heat generation and the rate of production of signaling molecules released into the circulation, this control mechanism allows the instant regulation of the single organ – the exercising skeletal muscle mass - that is the key determinant of the extent to which whole body homeostasis is threatened during exercise.

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Chapter 21

JIM MILLEDGE
HYPOXIA HONOREE 2007

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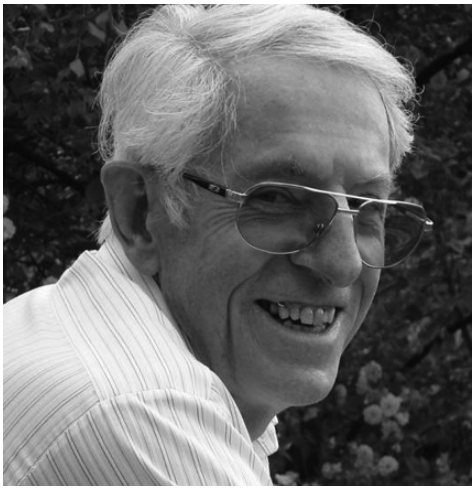

Abstract: Jim Milledge is well known to the international "Hypoxia" community for his many contributions to many high altitude medical and scientific expeditions, including the recent Extreme Everest Expedition in the aspring of 2007! His role as a physician, scientist, teacher, mentor and fried is cherished by those who have had the pleasure of working with him, either at home in the UK, or abroad during his many forays into thin air. In 2007 the International Hypoxia Symposium honored Jim for his outstanding service and role in leading the entire field of high altitude medicine and physiology.

Key Words: biography, high altitude physiology, mountain expeditions, gas exchange, cardiorespiratory physiology

It is a privilege to honor Jim Milledge at Hypoxia this year. For more than five decades he has contributed enormously to the field of high altitude medicine and physiology. His work has led to major advances in our understanding of the physiology of acclimatization to high altitude, particularly ventilatory changes, salt and water homeostasis, the exercise response to altitude and the comparative physiology of Sherpa

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people. His unbridled enthusiasm for science and expeditions is a great joy to those with whom he works, and continues to be a source of inspiration to many young people. Jim has taken part in more than a dozen major altitude research expeditions and held many leading roles including Medical Director of Northwick Park Hospital, President of the International Society of Mountain Medicine and honorary Professor of Altitude Medicine at University College London.

He has published widely with 75 original articles, 12 editorials and reviews, 10 book chapters, and most notably the seminal text 'High Altitude Medicine and Biology', co-authored with John West and Mike Ward and in its fourth edition this year.

Jim will highlight his major expeditions (Fig.2) and research contributions, but first I would like to share a little of his background. He was born in 1930 in China, and coming from a long line of missionaries working in far flung corners of the globe on both sides of his family, he was no stranger to adventure. He was raised in Tsang-Chow in northern China, before returning to the UK with his parents at the age of six. After a couple of years both his parents in turn were called to work in China and interned in Shanghai during the War, and so he was brought up by his aunt and uncle in North Wales with his two cousins. Together they shared a great passion for scaling sea cliffs and an appreciation of the spoken word, said to be a part of their Welsh heritage. He has retained this much valued gift of story telling to the present day such that a six week expedition will be brightened with a fresh story or song for every evening. He will gently remind anyone of the female gender who expresses delight at re-hearing a much loved story that 'a lady always hears a story for the first time!' After a narrow escape from being head-hunted to run a jam factory, he studied medicine at Birmingham University. During his university years he made maximum use of his time by joining the Air Squadron, and learning to ski and rock climb.

1960 Silver Hut expedition
1964 Sir Edmund Hillary's School's expedition
1977 North Wales
1979 Lake District
1980 Switzerland
1981 Mount Kongur in Xinjiang, China
1981 Mount Everest (AMREE)
1984 Pikes Peak, Colorado
1987 Mount Kenya
1989 Andes, Bolivia
1998 Kangchenjunga (Medical Expeditions)
2003 Makalu area (Medical Expeditions)
2007 Mount Everest (Xtreme Everest)

Figure 2. Major altitude research expeditions.

Jim graduated in 1954 and undertook his house jobs in Birmingham. During this year he married Betty, a fellow medical student. After house jobs, Jim's employment in the Royal Air Force took them to Hong Kong, where they both worked for two and a half years. Aviation medicine was perhaps Jim's first exposure to the effects of hypoxia on the body. The time in Hong Kong also gave them the opportunity to do voluntary medical work at a local clinic. They fortuitously met a Ghurkha officer during this period who informed them that Nepal had just opened it borders to visitors, and that it was a marvellous place to go trekking. In 1958 Jim and Betty travelled overland to Kathmandu, and then trekked to Pokhara. This was no mean feat at that time when few Westerners had passed that way before. In 1959 Jim and Betty returned to the UK, where Jim was a registrar in Southampton. Here he helped to set up a laboratory to perform lung function testing including spirometry and a crude test of gas transfer using a simple coal gas detector. He also persuaded the cardiology registrar to teach him to carry out arterial punctures, enabling him to perform the first arterial blood gases on respiratory patients in the hospital.

In 1960 to 1961 Jim took part in the land mark Silver Hut Expedition, which to this day remains the most comprehensive field expedition studying many aspects of altitude acclimatization. It was also the longest time an expedition of Westerners had resided at such high altitude. During the expedition Betty provided essential support for the team, as well as working at the Shanta Bhawan mission hospital in Kathmandu. On returning to England Jim busied himself writing up expedition findings, while the Principal of Vellore Hospital and Christian Medicine College in India happened to be in the UK for just 48 hours. The Principal arranged to meet Jim under the clock of Victoria station in London and an invitation was soon forthcoming to Jim and Betty to work in Vellore. This was a wonderful chance for Betty to pursue her interest in the medicine of developing countries, and Jim to further his career academic medicine. It is also a fine example of Jim taking his own advice to 'take the opportunity of a life time in the life time of the opportunity'. And so it was that they spent 10 happy years at Vellore, where Jim was a Lecturer, Reader and Associate Professor in Respiratory Medicine. He taught both undergraduate and post graduate students, worked as a respiratory physician, set up TB clinics, a lung function laboratory and arterial blood gas service, and worked closely with cardio-thoracic surgeons as they began open-heart surgery. He supervised technicians running the heart – lung pumps, and trained them to become independent. After this he began work on post-operative resuscitation and ventilatory support, and invented the 'Vellore – Ventilator'. Jim and Betty's children Maggie and John were born whilst they were in Vellore, and went to the local school in the compound. Family life didn't curtail their adventures, and the whole family enjoyed a trek to Everest base camp in 1970. Jim took leave from Vellore to spend time with John Severinghaus in San Francisco, during which time he carried out studies both in the laboratory at sea level and at altitude at White Mountain, and in the Peruvian Andes.

In 1972, Jim returned to England to become one of a number of young and dynamic consultants at Northwick Park Hospital near London. This busy District General Hospital was set up three years previously to serve the population of Harrow, and to study *common* diseases affecting large numbers of people, in contrast to some of the traditional famous teaching hospitals. Jim's appointment was a part time clinical and part

time academic post, enabling him to continue research into further aspects of clinical physiology, including the effects of hypoxia on the ECG, ventilation, heart rate and VO_2 max. During this time he carried out a number of further expeditions, including the famous American Medical Research Expedition in 1981. He became Medical Director of Northwick Park Hospital in 1992, before retiring from the National Health Service in 1995.

Sadly three and a half years before he retired, Betty died suddenly. However, it was with great joy a few years later he married Pat, a retired teacher and Head of Sixth form, with whom he shares other interests, Pat being a great linguist and musician. Over the past 10 years since Jim supposedly retired, he has been a wonderful leading light and mentor to hundreds of young enthusiasts of mountain medicine and physiology in the UK and elsewhere, who have benefited from his many gifts, summed up well by Brownie Schoene (Fig. 2). He continues to give extremely generously of his time, both with regular lecturing, and a willingness to share his pearls of wisdom and advise those embarking upon research projects. It is a great tribute to Jim that in the UK there are several young high altitude research groups, each of which he has been instrumental in mentoring and advising ~ Medical Expeditions, APEX (Edinburgh) and the Centre for Aviation, Space and Environmental Medicine (University College London). He has also overseen the setting up of the ISMM Mountain Medicine Diploma in the UK, and was the first honorary recipient of the diploma. These are certainly not 'arm chair' roles! He continues to enjoy walking in the hills and skiing, and just last month could be spotted in the French Alps burying and digging out victims while testing an avalanche jacket; soon he will be setting off on yet another 'last' expedition, this time to Everest. We are truly grateful to Jim for his wonderful mentorship – his passion for science and adventure will continue to live on through the many young people he inspires.

- Boundless positive energy and enthusiasm
- The physical and intellectual talent to pull things off
- A way of elevating those around him just by being who he is
- An ability to treat everyone with the same respect and encouragement
- Humility for his own history

Figure 3. Jim's gifts according to Brownie Schoene.

Figure 4. The dashing Jim back flying Tiger Moths after 40 years

Chapter 22

EXPLORING MOUNTAIN MEDICINE AND PHYSIOLOGY

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INTRODUCTION

I am honoured to be asked to give this talk to the Hypoxia Symposium. I have been fortunate to have attended all but one of the 15 symposia up to now including the first one in 1979. I feel inadequate to follow the illustrious names of Houston, Rahn, Pugh, Grover, Hultgren, Reeves, Severinghaus and West. I decided I should, like last time's Honoree, talk about my own experience of research in high altitude medicine and physiology. There will be some overlap with John West's talk of last time since we have been on two major expeditions together but that is a consequence of choosing us one after the other!

HOW DID I GET STARTED?

In November 1959 I read in the newspaper that Sir Edmund Hillary and Dr. Griffith Pugh were going to lead a scientific and mountaineering expedition the following year to the Everest region of Nepal to study the long term effects of high altitude. I wrote to Griff, having never met him, asking if there happened to be a place for me on his team. I was at the time a resident in Respiratory Medicine in Southampton and had done some climbing and skiing but had no expedition experience. He replied that the team was actually made up but if I was in London I should come and see him. Well I immediately asked for a day off and "happened to be in London" the next day! Someone dropped out and I was invited to join what subsequently became known as "The Silver Hut Expedition". Thus I found myself on my first major expedition and at the start of what became my professional hobby of Mountain Medicine and Physiology. Although my job, throughout my career, has been that of a general physician with a special interest in respiratory medicine, I have been fortunate to have been able to take time off to go on many expeditions to the great ranges, through the kind understanding of colleagues and family.

Hypoxia and the Circulation, edited by R.C. Roach *et al.*
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THE SILVER HUT EXPEDITION, 1960-61

The 1960-61 Himalayan and Scientific Expedition, to give it its official title, was a unique enterprise. It was dreamed up by Sir Edmund Hillary and Dr Griffith Pugh when they were together in the Antarctic. Griff Pugh had been with Ed on Cho Oyu in 1952, Everest in 1953 and in Antarctica in 1956/7. The idea for this long Himalayan expedition was based on the pattern, common in Antarctica, of leaving a party of scientists on the ice to "winter over". The idea was to study the long term effect of really high altitude on human lowland subjects. So the plan was to go out from Kathmandu after one monsoon, spend the whole winter at high altitude and in the spring to attempt an 8,000m peak, returning just before the next monsoon. Some members came only for the autumn, others for the spring part and some, including myself, were able to spend the whole nine months in the field. Our winter station was a pre-fabricated wooden hut, painted silver, which we set up at 5800m on the Mingbo Glacier in the Everest region of Nepal. This became known as the Silver Hut.

Figure 1. Sherpas carrying panels of the "Silver Hut" across a stream in the Khumbu,

Figure 2. the Silver Hut in place on the Mingbo Glacier at 5800 m with the fluted walls of the Mingbo Col behind. This was our home for the winter of 1960-61.

Our program of research included numerous studies in ourselves as we acclimatized. Many of these examined the changes which took place at the various points of the oxygen transport cascade from air to tissues. The project for which I was particularly responsible was on the changes in the chemical control of breathing with acclimatization (2). I was also involved in a large study of the ventilation, heart rate and cardiac output on exercise at various altitudes. This was especially Griff Pugh's interest. I also did a project on the changes in the ECG with increasing altitude.

We found that the height of 5800m was too high for optimum acclimatization. We all continued to be anorexic and to lose weight at this altitude. This weight loss was reversed by descent to Base Camp at the still considerable height of 4500m.

In the spring, some physiology was continued as we attempted to climb Mt Makalu (8481m). Exercise studies including measurement of VO2 max, were conducted up to the Makalu Col (7440m) by Mike Ward and John West. An account of this expedition with bibliography is given in reference 1.

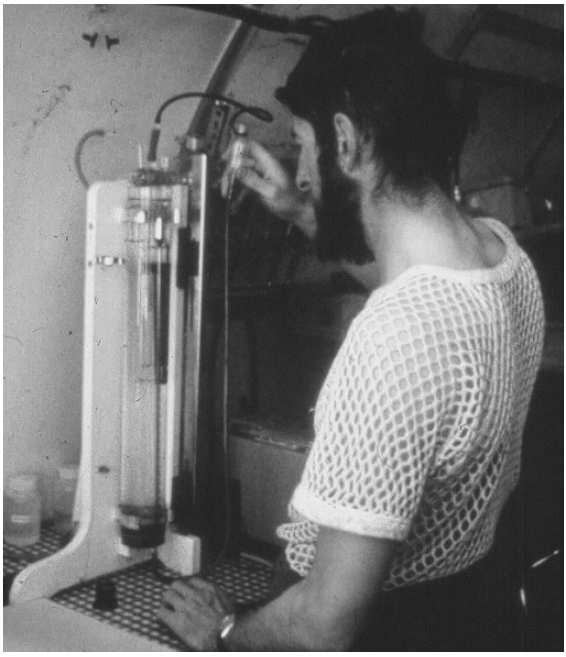

Figure 3. The author, inside the Silver Hut, using the Lloyd-Haldane apparatus to analyse alveolar gas samples for oxygen and carbon dioxide after a control of breathing experiment.

AFTER "SILVER HUT"

Immediately after the Silver Hut Expedition I came back to Oxford where Dan Cunningham and Brian Lloyd, my mentors in the control of breathing project, were based in the Physiology Department. They were hosting the Haldane Centenary Symposium that summer, honouring the birth of JS Haldane the great Oxford physiologist. All the leading cardio-respiratory physiologists were there. One of Haldane's classic contributions was in the field of control of breathing and altitude. He led a famous expedition to Pikes Peak in 1911, so our work, "hot off the press" was very well received. It was

my first scientific presentation. I decided to try and make a career in academic medicine and was thinking of applying for a post in a British medical school when we received a pressing invitation to join the staff of Christian Medical College, Vellore in South India. My wife, Betty, as an anesthesiologist was probably even more welcome. We worked there for the next ten years with a year's sabbatical, in the middle, in San Francisco. There I had a research fellowship working with John Severinghaus.

In 1964 Dr. Sukhamay Lahiri, who had been on the Silver Hut Expedition, invited me to join him in a small physiological team as part of Sir Edmund Hillary's Second Schoolhouse Expedition to Solo Khumbu, Nepal. While Ed and his team built two schoolhouses, a bridge and the Lukla Airstrip as part of his aid to Sherpas, we studied the differences between ourselves, lowlanders and Sherpa highlanders at a camp high above Lukla at 4880 m. We found that Sherpas had a much lower hypoxic ventilatory response than did lowlanders both at rest and exercise (3,4). John Severinghaus and colleagues almost simultaneously found the same thing in Andean highlanders.

Figure 4. 1964, Our Physiology Camp on the ridge above the Inuku Valley, 4600 m. An exercise experiment with a Sherpa subject (Hakba Tsering). The large plastic Douglas bag provides inspired gas with either increased or decreased oxygen in order to test the hypoxic ventilatory response.

THE SEVENTIES

In 1972 we returned from India and I was fortunate in getting a job at Northwick Park Hospital where the Medical Research Council had established its Clinical Research Centre. There I began working in Dr. John Nunn's division of anaesthesia. A year later I got a combined MRC and NHS appointment. In the '70s we were unable to get to the Great Ranges but did a series of field studies on the effect of long continued exercise (hill walking) on fluid balance and related hormones. These were stimulated by the accounts of high altitude pulmonary edema in which strenuous exercise seemed to be a risk factor. We first studied the effect of hill-walking at low altitude (< 1000m in the hills of the UK) on fluid balance and related hormones. Then we repeated the stud-

ies in Switzerland adding altitude to the exercise. We found the effect of abrupt change from semi-sedentary to exercising life style was to retain some water, a lot of sodium and increased plasma and extra-cellular fluid volumes (5,6).

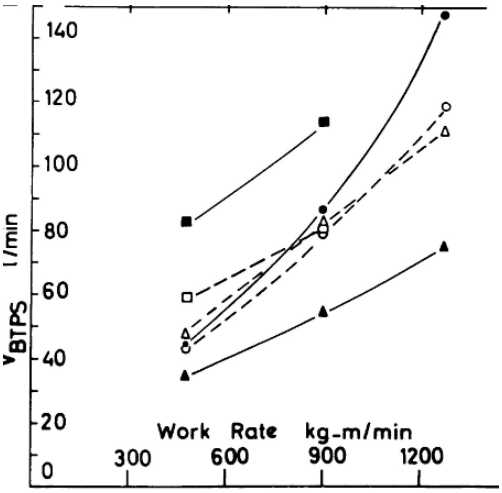

Figure 5. The result of exercise tests at increased work rates on a lowlander, closed symbols and full lines and a Sherpa, open symbols and dashed lines. The three lines are when breathing air, middle line, sea level PO2, lower line and decreased PO2, upper line. It will be seen that while these changes in inspired PO2 result in a marked change in ventilation in the lowlander, there is very little change of ventilation in the Sherpa

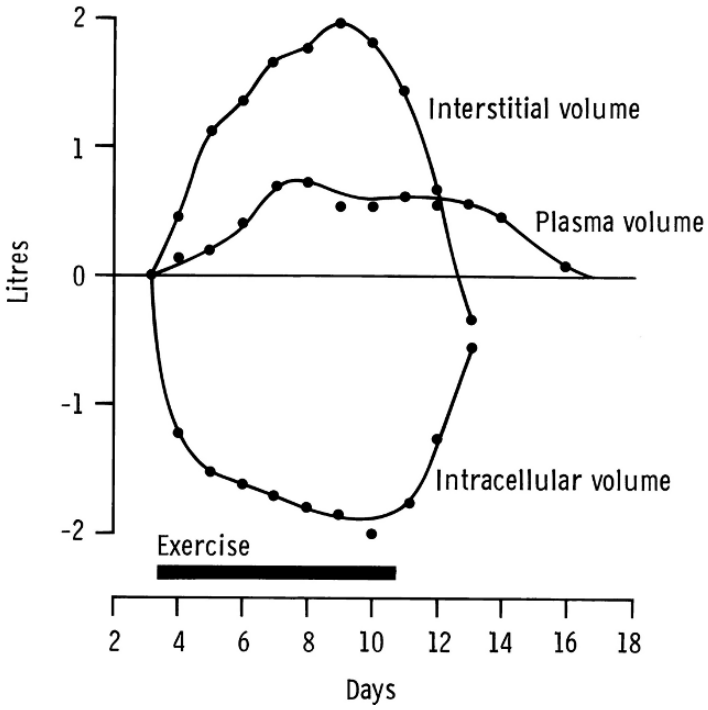

Figure 6. Graph showing the calculated changes in various fluid compartments, induced by hill walking, 8 hours a day for 7 consecutive days. There is a modest water retention (~600 mL) but a large sodium retention (~360 mmols). There was no significant change in serum sodium so water moved out of the intra-cellular into the extra-cellular compartment, expanding the interstitial and plasma volumes, resulting in dilutional anaemia.

THE EIGHTIES

In 1981 I was invited by Mike Ward, whom I had known from the Silver Hut Expedition, to join him and a team of four elite climbers led by Chris Bonington who were attempting to make the first assent of Mt. Kongur (7719 m) in Xinjiang (China). We compared the climbers with us more averagely fit scientists and found evidence that their physiology had moved some way towards that of Sherpas, in that their HVR on exercise was lower than ours. Also on this expedition we collected blood samples for the analysis of erythropoietin. The immunological method had recently become available and was being carried out by Mary Coates in the CRC. The figure in our paper of 1985 showing the altitude, hematocrit and Epo levels against days of the expedition has been reproduced more than any other illustration of my work (7).

Also in 1981 I had been invited to join John West's American Medical Research Expedition to Everest (AMREE). Twenty years after Silver Hut we extended much of the work of that expedition with studies on exercise and gas exchange including alveolar gas measurements up to the summit. There were many other projects in this expedition including sleep studies at Base Camp and in the Western Cwm. I looked at the angiotensin response to renin which I found to be even more blunted than at more modest altitude in Switzerland.

In 1987 and '89 I had two expeditions with the Royal Navy Mountaineering Club to Mount Kenya and to Bolivia. On these trips we studied fluid and salt balance in relation to acute mountain sickness (AMS). We showed that there was a correlation between sodium retention and aldosterone levels on the day of ascent and the subsequent AMS scores. We also showed that there was no correlation between AMS and fitness (VO_2max) or with the hypoxic ventilatory response. These studies took place during the first 4-5 days at altitude and then we went off in twos and fours to climb a number of peaks.

HIGH ALTITUDE MEDICINE & PHYSIOLOGY: A TEXTBOOK

Mike Ward, who sadly died in 2005, had been the prime mover of the 1951 Everest Reconnaissance Expedition which found the route from the South. He had been Medical Officer for the 1953 Everest Expedition and had also been in the Silver Hut (when he and three others made the first ascent of Ama Dablam). He had also been one of the team on our four hill-walking fluid balance studies as well as leader of the Kongur Expedition. In 1975 he had published the first ever text book on Mountain Medicine and I had helped with some of the chapters. In the mid eighties I asked him if he was thinking of a second edition. He invited me to join him as co-author and we later recruited John West so that in 1989 we published the first edition of our textbook, "High Altitude Medicine and Physiology". There have since been two more editions (in 1995 and 2000) and we have now revised it for the fourth edition, with "Brownie" Schoene taking Ward's place as third author. It will be published this year.

THE NINETIES

I had a number of treks or minor expeditions to the Himalayas of Nepal and India in 1991, '94, '96 and to the Karakoram in '95. In August '95, on my 65th birthday I retired from the NHS and MRC and could now spend more time on Mountain Medicine.

In 1992 I became involved with a group of young British doctors who mounted an Everest Expedition in 1994. This group has evolved into the charity, "Medical Expeditions". We have had two further major expeditions in 1998 to Kangchenjunga and in 2003 to Chamlang Base Camp, all in Nepal. The pattern of these quite large medical research expeditions has been to have a small climbing group attempting the major peak and up to about 50 trekking members going to the base camps in small groups. The science has been done in London before departure and at the base camps with some simple observations on the trek.

Medical Expeditions has two charitable aims, to support research and education in altitude medicine and physiology. The research has mostly been done on the major expeditions and the educational aim has been covered by running week-end courses in Mountain Medicine at intervals in North Wales for the past 14 years. Four years ago we began offering courses for a Diploma in the subject. This was the first such course in English, though courses in Europe have been running for a number of years.

CONCLUSION

I have been so very fortunate to have been involved with our subject for so long. I have seen incredible changes in the technology available to us and considerable advances in both the physiology of high altitude and understanding of the medical conditions of mountainous regions. My own contribution to these has been very small. I hope that I have been able to disseminate these advances by talks, lectures and writing; contributing, I hope, to a wider understanding of the subject and possibly fewer deaths. My main delight, however, has been the many friends I have made through mountaineering, expeditions, conferences etc. and the abiding memories I now have over this long time.

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Chapter 23

CARLOS MONGE CASSINELLI: A PORTRAIT

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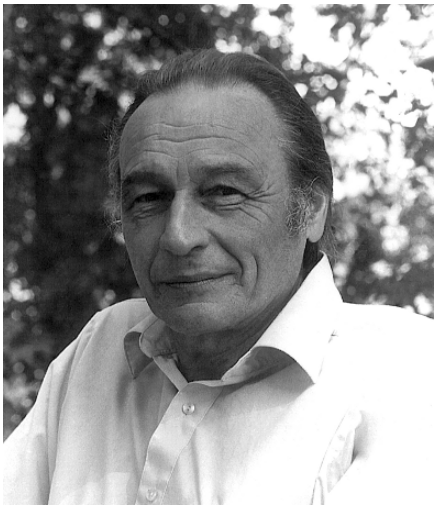

Abstract: Carlos "Choclo" Monge Cassinelli, a pillar of scientific integrity and great friendship to high altitude researchers throughout the world passed away in 2006, and was honored by his many friends at colleagues at the 2007 International Hypoxia Symposium. Choclo had more than 600 publications to his name, in fields diverse from his medical specialty in renal disease, to the biology of animals adapting to the high altitudes of South America. Those of us who had the pleasure of working with Choclo will always remember the sparkle in his eye, the intelligent, probing questions, and his tremendous sense of humor. He was recognized as a world authority on high altitude dieases, with particular accolades for his work invloving high altitude resident populations. This tribute and picture gallery pay tribute to Choclo, written by Fabiola Leon Velarde and Jean Paul Richalet.

Key Words: biography, altitude pathology, altitude residents, Monge's disease

Hypoxia and the Circulation, edited by R.C. Roach *et al.*
Springer, New York, 2007.

Carlos Monge Cassinelli (Choclo) deserves a special position in the history of science and medicine in Perú and abroad. Son of Carlos Monge Medrano, discoverer of Chronic Mountain Sickness (Monge's Disease) in 1921, he was immersed since he was a boy in the world of the study of the people of the Andes, which probably weighed decisively on the direction of his vocation in medicine.

Choclo was born in Lima (1921), and received his medical education in the National University of San Marcos (UNMSM) between 1938 and 1947. As a medical student, he worked in human physiology with Alberto Hurtado, (Director of Research of the Institute of Andean Biology at UNMSM and founder of the Cayetano Heredia Institute of High Altitude Research, in 1961, where they studied in the inhabitants of Morococha (4,540 ms).

In this stage, he confirmed preliminary work that showed a diminution of the concentration of glucose in blood in basal conditions and of the lactic acid concentration in conditions of exercise, in comparison with sea level. The most important contribution of this work is that he presents for the first time what later would be known as "the lactate paradox ", still today a matter of study by the international scientific community. Since 1950 he investigated circulatory dynamics (using the dilution technique of Evans Blue with his student, friend and colleague, Dr. Alberto Cazorla. They studied people from sea level compared with Andean high altitude subjects, finding a significant increase of the pulmonary volume in the inhabitants of Morococha in comparison with those of Lima.

In 1953 he spent a year in the Cleveland Clinic as a medical investigator. There he developed a method for the measurement of hexametonium in blood, showing its extra-cellular distribution. When he returned to Peru he developed an intense study of the renal function in the high altitude dweller, producing the predominant number of contributions in this field in the international literature. His work shows an inverse correlation between the hematocrit and the renal plasma flow, and between the hematocrit and the filtration fraction, with a conserved auto-regulation function of the glomerular filtration. He demonstrated that in spite of these glomerular changes, the tubular functions of concentration, osmotic diuresis, bicarbonate and proton elimination were normal. To date, these contributions are still very important for the renal physiology of the Andean native.

His sense of humor was legend, he was always ready with a story or a joke to enjoy the conversation. His students and collaborators knew from anecdotes and stories what he thought was important in the study of high altitude polycythemia. With this topic, Choclo reinitiated the research he had carried out as a medicinal student, integrating the team of Alberto Hurtado. His more important contribution in this field has been to show that the hematocrit rises significantly with age at high altitude. He proposed the possibility that Chronic Mountain Sickness is a lack of adaptation of the population to high altitude, and not a clinical entity that affects only some individuals. He demonstrated with his collaborators that the normal diminution of ventilation with age is associated with an increase of the hematocrit, adding a possible causal relation between the fall of the ventilation with age and the corresponding elevation of the hematocrit. This contributed in a substantial way to the recognition of this disease as a problem of public health for the mountainous regions of the world. He had a great inclination for

mathematical models, through which he defined in a quantitative way the physiopathological mechanisms that give rise to the appearance of Chronic Mountain Sickness. He quantified and put under a mathematical model also the concept of concentration of optimal hemoglobin for life at high altitudes, a concept corroborated and accepted now by other authors.

He has always remarked and called attention to high altitude erytremia as an unsuitable adaptation and to the physiological design of Andean physiology that he believed corresponds to the design of sea level physiology. His classic log-log graph, relating the P50 to the animal weight, allows to differentiate two groups of animals, and place the high altitude human as belonging to the group of sea level animals, thus, as only showing phenotypic adaptations. These results can be extrapolated to the domestic animals that the Spaniards brought to South America and introduced in the Andes.

In order to prove if a hemoglobin with a high affinity for oxygen, found in mammals and birds genetically adapted to high altitude, is also present in ectotherms (whose adaptation to cold favors the high altitude adaptation), Choclo and his colleagues studied 3 subspecies of *bufo* from sea level, 3,100 and 4,540 m. They found that the hemoglobin affinity for oxygen is higher in the *bufos* from high altitude, even at sea level temperatures. At its normal ecological temperatures, the hemoglobin affinity of the bufo adapted to 4540 m of altitude increases greatly. This work reveals the sensitivity of hemoglobin to undergo genetic changes even at moderate hypoxic atmospheres, and demonstrates, for the first time that anurans show adaptations to the high altitude environment.

Choclo liked very much compared physiology, and field studies in the Andes. One of his favorites was the study in which he and his colleagues showed that the air chamber of the Andean gull egg has CO_2 and O_2 pressure values similar to those in the pulmonary alveoli of human residents at equivalent altitudes. These findings persuaded him that the diffusive phenomenon that occurs through the eggshell is sufficient to establish the values that appear in adult mammals and birds using pulmonary breathing. He considered that the pulmonary convective process rather adjusts, and does not originate, the basal numbers of partial pressures of alveolar gases, a concept of some importance for the evolution of the respiratory system.

Choclo has managed to transfer the information of comparative physiology of the natural adaptation to high altitude to the epidemiology of Monge´s Disease, obtaining therefore an integration of the knowledge from basic physiology to medical studies in Chronic Mountain Sickness. In more universal and integrative terms, because the capacity to integrate knowledge was one of his greater virtues, Choclo had the vision to approach with a fine perception all the subjects that matter in high altitude research, i.e., the micro and macro mechanisms of tolerance to hypoxia, the physiological and clinical aspects of adaptation to high altitude, the limits of that adaptation, as well as the interrelation between these fields of study. His fields of interest go from cellular models to more organismic models like fish, amphibians, birds and rodents, and to the problems of high altitude human public health. The National Prize of Sciences of Canada, and well recognized biochemist, zoologist and Choclo's close friend, Professor Peter Hochachka said it well: "Choclo has introduced the intellectual thread in the eye of the needle necessary to integrate all the physiology of high altitude, and to generate, from

this integration, a new knowledge with greater added value".

Carlos Monge C, was Rector (1970) of Cayetano Heredia University (Universidad Peruana Cayetano Heredia –UPCH-), the university which he contributed to founding. He arrived in that position with great support, as a demonstration that the university felt he was the legitimate successor of the "Peruvian Masters". Nevertheless, he evaded this type of activity (administrations), that moved him away from his laboratory, and for that reason, within two years after his election, Choclo resigned this position, accepting the invitation as Visiting Professor at Cambridge University (U.K.). This action, unique in our country, gives account clearly of his priorities.

For his work, Carlos Monge has received, throughout his life, multiple recognitions, of these, it is worth mentioning: The National Award for Science, Perú (1972), he has been Fellow of the Churchill College, U.K. (1973), Honorary Member from the American College of Physicians (1978), Scholar of the National Institute of Health (1979), Honorary Member from the University of Alabama (1980), and from the Academy of Medicine, Chile (1983), he has received the "Palmas Magisteriales en el Grado de Amauta" from the Peruvian Government (1992), he was Fellow of the John Simon Guggenheim Memorial Foundation (1994). Additionally, in 1995, the IX International Hypoxia Symposium, in Lake Louise, Alberta, Canada was dedicated to Carlos Monge Cassinelli. In 2000, Carlos Monge has in charge the inaugural Conference of the IV World Congress of High Altitude Medicine and Physiology; in Arica-Chile, and the same year he received the Gold Medal, from the Institute Foundation Hipólito Unanue. In 2003, he was awarded with the National Award for Sciences from the Peruvian National Council of Sciences and Technology (CONCYTEC).

As a member of more than 20 international expeditions to regions like the Peruvian, Bolivian and Chilean Andes, in numerous opportunities, as well as to the Himalayas in Nepal, Carlos Monge C. was an universal man, nevertheless, he knew and stated very clearly that although the international actors are necessary for their knowledge and their advanced technology, they should not replace the local knowledge, which accumulates unique experiences for the advancement of science, and that it is there, in our capacities, where the potential for the development of Peru resides. Carlos Monge C. instructed more than a generation of students, that the task of doing research in Peru is not easy, but that it is possible and essential. He also taught that the compensation of the scientific work is not at the end of the way, but in the pleasure to be crossing it day to day with passion and devotion.

One recent statistic report showed that in the last three years, Peru published, each year, in international journals, approximately 600 original articles and 30 review articles in the areas of Medical and Biological Sciences. In these years, the "Universidad Peruana Cayetano Heredia" generated 30% to 40% of those publications, with almost 10% belonging to the laboratory of Carlos Monge C. This is the best way to render tribute to Choclo, my teacher, the colleague, and the best friend.

Figure 2. The Monge's family : sitting in front : Doña Cristina Cassinelli (Choclo's mother) and Carlos Monge Medrano (Choclo's father). Choclo is standing in the back, in the center.

Figure 3. Choclo with a patient suffering from Chronic Mounatin Sickness in his laboratory at Cerro de Pasco (4300m), Peru.

Figure 4. Blood sampling from a llama at Morococha with Robert Winslow.

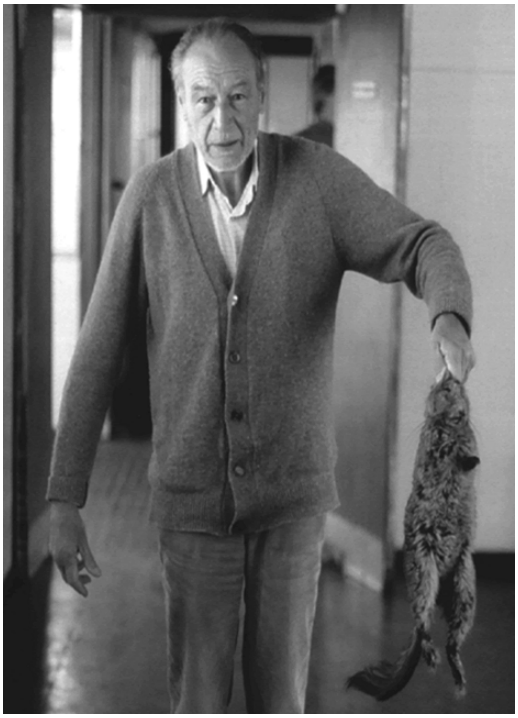

Figure 5. Choclo bringing "viscachas" to the laboratory in Morococha, Peru.

Figure 6. Choclo and Fabiola León-Velarde in his lab.

Figure 7. Arguing with Peter Hochachka and Jean-Paul Richalet

88. CEREBRAL AUTOREGULATION IS TRANSIENTLY IMPAIRED IN ACUTE MOUNTAIN SICKNESS; MODULATION BY PHYSICAL EXERCISE. PN Ainslie[1], K Evans[2], DM Bailey[2]. *[1]Department of Physiology, University of Otago, Dunedin, New Zealand, [2]Department of Physiology, University of Glamorgan, UK. Email: philip.ainslie@stonebow.otago.ac.nz*

The current study examined if increased intracranial pressure (ICP) secondary to an impaired cerebral autoregulation (CA) is implicated in the pathophysiology of acute mountain sickness (AMS). Nine males who developed clinical AMS (AMS+) were compared to 9 males who remained healthy (AMS-). Estimated ICP (eICP) and CA were assessed at rest in normoxia, after 6h passive exposure to hypoxia [12% oxygen (O_2)], immediately following a cycling test to exhaustion and following a further 2h of passive recovery in hypoxia. A dynamic rate of CA was calculated in two ways: 1) from continuous recordings of blood flow velocity in the middle cerebral artery (MCAv) and arterial blood pressure (BP) during a transient episode of hypotension; and 2) using transfer-function gain and phase shift in mean BP and MCAv. Estimated ICP was calculated from the difference between mean BP and estimated cerebral perfusion pressure, which was derived from the difference between mean BP and calculated zero-flow pressure. In AMS+, eICP increased after 6h hypoxia and CA was impaired - apparent in both a reduction in the MCAv to return to normal during transient hypotension and in elevation in the low-frequency gain between MCAV and mean BP ($P < 0.05$ vs. normoxia); this impairment in CA was consistently related to the increase in AMS and associated headache scores ($r = -0.66$ to -0.70, $P < 0.05$). While acute exercise did not alter CA or eICP, symptoms improved in AMS+ but became worse in AMS-. These findings establish impaired CA and elevated eICP as intracranial risk factors that predispose to severe AMS during passive but not active exposure to hypoxia.

89. UTERINE AND ILIAC ARTERIAL BLOOD FLOW IS ASYMMETRIC IN ANDEANS BUT NOT EUROPEANS AT HIGH-ALTITUDE. VA Browne[1], L Toledo-Jaldin[4], RD Davila[3,4], LP Lopez[4], E Vargas[4], A Rodriguez[4], M Aguilar[4], D Cioffi-Ragan[2], LG Moore[1,3]. *[1]Altitude Research Center, Dept of Surgery, Div. Emergency Medicine UCDHSC Denver, CO, [2]Dept. Obstetrics and Gynecology UCDHSC Denver, CO, [3]Dept. Health/Behavioral Sciences UCDHSC Denver, CO, [4]Instituto Boliviano de Biología de Altura, La Paz, Bolivia. Email: Vaughn.Browne@uchsc.edu*

OBJECTIVES: Andeans (AND) with preeclampsia (PE) and Europeans (EUR) residing at high-altitude exhibit reduced uterine artery (UA) but increased lower body blood flow (see other abstracts). We asked whether these changes occur equally in right (R) and left (L) uterine, common (CI), and external iliac (EI) arteries. METHODS. We studied 155 AND, 32 AND with PE (AndPE), and 38 EUR between 20–36 wk gestation. Using Doppler ultrasound, we calculated blood flow (Q) as mean velocity (TAM) x vessel cross-sectional area. RESULTS. In normal AND, TAM and Q were ~25% higher in the left CI and UA ($P<0.001$), although their diameters were symmetric. In contrast, TAM was equal in both EI, but the left was ~12% smaller. The ratio of EI/UA Q was ~40% lower, compared to the right ($P<0.0001$), confirming higher L UA Q. In AndPE, CI and UA diameters, TAM, and Q were symmetric. However, the ratios of CI/UA and EI/UA Q were 25% and 1.5-fold higher on the left, respectively, indicating greater reduction in LUA Q. Compared to AND, there was a ~3.5 fold reduction in L UA Q, mainly due to greatly

diminished end diastolic Q. In EUR, diameters, TAM, and Q were equally reduced in the three arteries studied, compared to AND. Symmetrically increased ratios of CI/UA and EI/UA diameter were consistent with disproportionately small UA. CONCLUSIONS. It is unclear why asymmetric blood flow was present in Andeans. UA blood flow was inversely related to EI diameter, suggesting that varying the ratio of EI/UA diameter is a key adaptive mechanism for regulating blood flow distribution during pregnancy. The more pronounced reduction in L vs. R UA blood flow in preeclampsia suggests that incomplete trophoblast remodeling of maternal end arteriolar vessels is asymmetric. (NIH-HL079647-01S1).

90. PREECLAMPSIA IN ANDEANS AND EUROPEAN ANCESTRY REDUCE UTERINE ARTERY BLOOD FLOW AND FETAL GROWTH AT HIGH ALTITUDE BY DIFFERENT MECHANISMS. VA Browne[1], L Toledo-Jaldin[4], RD Davila[3,4], LP Lopez[4], E Vargas[4], A Rodriguez[4], M Aguilar[4], D Cioffi-Ragan[2], LG Moore[1,3]. *[1]Altitude Research Center, Dept of Surgery, Div. Emergency Medicine UCDHSC Denver, CO, [2]Dept. Obstetrics and Gynecology UCDHSC Denver, CO, [3]Dept. Health/Behavioral Sciences UCDHSC Denver, CO, [4]Instituto Boliviano de Biología de Altura, La Paz, Bolivia. Email: Vaughn.Browne@uchsc.edu*

OBJECTIVE. Compared to healthy Europeans (EUR) residing at high altitude, babies born to normal Andeans (AND) are relatively protected from reduced birth weight. We asked whether preeclampsia (PE) and EUR ancestry influence birth weight by different physiologic mechanisms. METHODS. We studied 155 AND, 32 Andeans with PE (AndPE), and 38 EUR between 20–36 wk gestation. Using Doppler ultrasound, we calculated blood flow as mean velocity x vessel cross-sectional area. Babies weighing below the 10th percentile adjusted for age and sex were classified as small for gestational age (SGA), and pre-term if <37 wk. RESULTS. PE and EUR ancestry increased the frequency of SGA babies (AndPE=44%, EUR=23%, vs. AND=15%, $p<0.001$), and pre-term births (AndPE=28%, EUR=16%, vs. AND=5%, $p<0.001$). Peak flow velocity in umbilical arteries (UMBA) was 15% lower, but the ratio of middle cerebral to UMBA peak flow was greater in AndPE and EUR fetuses compared to AND ($p<0.001$). Head circumference was similar in all groups, indicating preservation of fetal brain blood flow. Uterine artery (UA) blood flow was 6-fold lower in EUR compared to AND due to smaller UA diameter and slower velocity ($p<0.0001$). In AndPE, UA diameter was unchanged from AND but slower mean velocity resulted in 3 fold lower blood flow, with differences diminishing near term. CONCLUSIONS. In EUR and AndPE at high altitude, reduced UA blood flow restricts fetal growth, but fetal brain blood flow is preserved. EUR ancestry and PE decrease UA blood flow by different mechanisms. We speculate that genetic adaptations in Andeans result in increased UA diameter thereby permitting higher mean velocity. However, incomplete trophoblast remodeling of maternal end arteriolar vessels in preeclampsia may be due to defects emanating from fetal factors. (NIH-HL079647-01S1).

91. INFLAMMATORY "PRIMING" PREDISPOSES TO ACUTE MOUNTAIN SICKNESS. Damian Bailey[1], Sarah Taudorf[2], Ronan Berg[2], Carsten Lundby[3], Bente Pedersen[2], Kirsten Moller[2], [4]. *[1]Department of Physiology, University of Glamorgan, UK, [2]Centre of Inflammation and Metabolism, Department of Infectious Diseases, Rigshospitalet, Denmark, [3]Copenhagen Muscle Research Centre, Rigshospitalet, Denmark, [4]Department of Cardiothoracic Anaesthesia, Rigshospitalet, Denmark. Email: dbailey[1]@glam.ac.uk*

The present study examined whether a standardized inflammatory stimulus would increase the incidence and severity of AMS in human volunteers exposed to inspiratory hypoxia. Following ethical approval and informed consent, thirty-six subjects were randomly assigned single-blind to one of three interventions; [1] passive exposure to normoxia for 12h with intravenous endotoxin infusion (0.075ng/kg/hr) between 4 to 8h (N-LPS, n = 12); [2] normobaric hypoxia

(12.9% O_2) for 12 h with endotoxin infusion (H-LPS, n =13); and [3] hypoxia with saline infusion (n = 11, H-SAL). AMS was recorded at baseline in normoxia (0h) and following 4h and 9h exposure using the LL and ESQ-C scoring systems. Clinical AMS was diagnozed as a combined LL and ESQ-C score of ≥5 and ≥0.7 points respectively following 9h. Data were analyzed using Kruskal-Wallis and a posteriori Bonferroni-corrected Mann-Whitney U tests with data expressed as mean ± SD. Endotoxin (N-LPS) catalysed mild increases in LL (0h: 0 ± 1 vs. 4h: 1 ± 1 vs. 9h: 2 ± 2) and ESQ-C (0.000 ± 0.000 vs. 0.051 ± 0.083 vs. 0.345 ± 0.348 points) scores that were less pronounced than hypoxia alone (H-SAL: 0 ± 0 vs. 2 ± 2 vs. 3 ± 2 and 0.000 ± 0.000 vs. 0.664 ± 0.618 vs. 0.730 ± 0.683 points, $P < 0.05$). The greatest increase was observed in H-LPS (0 ± 0 vs. 2 ± 2 vs. 5 ± 3 and 0.018 ± 0.061 vs. 0.674 ± 0.786 vs. 1.348 ± 0.933 points, $P < 0.05$). Clinical AMS was diagnozed in 85% of H-LPS compared to 0% and 9% in N-LPS and H-SAL groups, respectively. These findings suggest a role for systemic inflammation in the pathophysiology of AMS. Acknowledgements: SRIF-III, National Research Council (File #22-04-013), Copenhagen Hospital Corporation (File #145-13).

92. INTERMITTENT SIMULATED HYPOXIA VIA RE-BREATHING IMPROVES CYCLING PERFORMANCE. CJ Babcock, TE Kirby. *Sport and Exercise Science, Physical Activity and Educational Services, The Ohio State University. Email: babcock.11@osu.edu*

PURPOSE: To quantify the effects of intermittent simulated altitude exposure via re-breathing on cycling performance. METHODS: Eighteen, well-trained cyclists use a re-breathing device for 15 days. Subjects were blind and randomly assigned to a “low altitude” constant exposure group (CON) or progressively increased “high altitude”group (TRT). Each exposure alternated between hypoxic and atmospheric air for 6 min and 4 min, for 1 hour/day. Oxygen saturation was held constant (98% over 15 days) for CON or progressively reduced (90% on the 1st day to 77% on the 15th day) for TRT. Performance tests measured familiarization to the protocol (FAM), prior to simulated altitude exposure (PRE) and following simulated altitude exposure (POST) using the critical power protocol to examine power output in a 15 minute and 3 minute time trial effort. Measurements of lactate, oxygen consumption, heart rate, hematocrit and reticulocytes were examined. RESULTS: There was a significant power output improvement (3%) for TRT at POST 15 minute time trial (FAM = 322.6 ± 12.2 watts, PRE = 325.0 ± 12.2 watts, POST = 335.0 ± 11.9 watts), and no significant difference in the CON group. This was a significant between-subjects effect ($p=0.011$). There were no significant differences in the power output for the 3 minute time trial at POST. Despite a trend of increased hematological characteristics in the TRT group, results were not significant. A significant decrease ($p=0.021$) in Heart Rate Index (HRavg/wattavg) was revealed for the TRT group at POST (PRE = 0.564 ± 0.044; POST = 0.544 ± 0.053). CONCLUSIONS: In well trained cyclists, progressive hypoxia using a re-breathing device revealed an improved power output in a highly aerobic powered cylcing event. These findings are similar to performance adaptations reported in other acclimatization studies, terrestrial or simulated.

93. OPTIC DISC SWELLING AT HIGH ALTITUDES. Martina M. Bosch[1], Daniel Barthelmes[1], Tobias M. Merz[2], Konrad E. Bloch[3], Alexander J. Turk[3], Urs Hefti[4], Florian PK Sutter[1], MG Wirth[1], Marco Maggiorini[5], Klara Landau[1]. *[1]Ophtalmology Kinik, University Hospital Zurich, Switzerland, [2]Intensive Care Unit, University Hospital Bern, Switzerland, [3]Pneumology, University Hospital Zurich, Switzerland, [4]Department of Surgery, Kantosspital, Aarau, Switzerland, [5]Intensive Care Unit, University Hospital Zurich, Switzerland. Email: klinmax@usz.unizh.ch*

Aim: We sought to determine the incidence of optic disc swelling as a possible indicator for cerebral edema in healthy mountaineers exposed to very high altitudes and to correlate these

findings with clinical and environmental parameters. Method: 27 mountaineers were included in a study during an expedition to Muztagh Ata (7546m/ 24, 757ft). Examinations including fundus photographs, visual function tests, cerebral acute mountain sickness (AMS-c) scores, oxygen saturation and blood pressure were performed 1 month prior and 4.5 months after the expedition at 400m (1, 300ft), and during the expedition at 4497m (14, 754ft), 5533m (18, 153ft), 6265m (20, 554ft) and 6865m (22, 523ft). The optic disc photographs were allotted to three groups: no swelling, possible swelling, and swollen disc. Results: 16 out of 27 (60%) climbers had disc swelling; oxygen saturation values were 57%-100%; AMS-c scores between 0-2.4. Optic disc swelling showed negative correlation with oxygen saturation (r=-0.27, p=0.01) and positive correlation with AMS-c score (r=0.2, p=0.02) Oxygen saturation is a strong predictor for optic disc swelling (Wald Stat.=5.03, p=0.02). Visual function tests and blood pressure did not show significant changes. Conclusion: Optic disc swelling has a high incidence in mountaineers climbing at high altitudes and correlates with the extent of hypoxia and the severity of AMS symptoms. Optic disc swelling appears to be associated with possible changes within the central nervous system during AMS. Whether it is the expression of increased intracranial pressure with or without mild high-altitude cerebral edema remains to be determined. Funding: Swiss National Science Foundation Grant, Pfizer Research Grant.

94. ALTO$_2$LAB, A SIMPLE, HAND-HELD, RE-BREATHING DEVICE, ACHIEVES ACCURATE HYPOXIC CONDITIONS AT LOW COST. RA Backhaus[1], R Cote[1], CJ Babcock[2]. *[1]Pharma Pacific, Phoenix, AZ Sport & Exercise Sci, Ohio State Univ, Columbus OH. Email: rabackhaus@pharmapacific.com*

Exposure to controlled hypoxic conditions mimicking high altitude is an accepted method of training for endurance athletes. Hypoxic preconditioning is also being tested for its potential therapeutic benefits in protecting cardiac, respiratory and neurological pathways against ischemic insult. These discoveries have heralded a need for a reliable, inexpensive, and easy-to-use hypoxic conditioning device that is portable, hygienic and does not require external power. One such device is AltO_2Lab, a hand-held, re-breather that simulates hypoxic conditions of altitudes in excess of 7, 000 m (22, 000 ft). The AltO_2Lab system uses a canister containing a CO_2 absorbent and a series of air mixers to achieve the desired target altitude for users who continuously monitor internal blood O_2 levels with a pulse oximeter. Athletes currently use AltO_2Lab for intermittent hypoxic training (IHT) protocols. Subjects alternate 6 min of hypoxic breathing through the device with 4 min of breathing room air, six times, for a total of 56 minutes per session. To complete the "standard" IHT program one session is performed each day for 15 days, by progressively increasing the hypoxic stress each day from 93% to 75% blood O_2 saturation. Until now, there was uncertainty in quantifying the precise hypoxic dosage each user experienced. However, newly available wrist-recording pulse oximeters with accompanying software now enable the AltO_2Lab to generate precise hypoxic dosages tailored to each individual's needs. This advance allows administration of precise hypoxic dosages with AltO_2Lab at a fraction of the cost of existing technologies. AltO_2Lab was originally developed in New Zealand for their Olympic training program, to enable all endurance athletes to experience affordable altitude training without the hassle of relocating to high elevations or sleeping in expensive altitude tents.

95. EUROPEANS AT HIGH-ALTITUDE AND PREECLAMPTIC ANDEANS REDISTRIBUTE BLOOD FLOW TO FAVOR THE LOWER BODY BY DIFFERENT MECHANISMS. VA Browne[1], L Toledo-Jaldin[4], RD Davila[3,4], LP Lopez[4], E Vargas[4], A Rodriguez[4], M Aguilar[4], D Cioffi-Ragan[2], LG Moore[1,3]. *[1]Altitude Research Center, Dept of Surgery, Div. Emergency Medicine UCDHSC Denver, CO, [2]Dept. Obstetrics and Gynecology UCDHSC Denver, CO, [3]Dept. Health/Behavioral Sciences UCDHSC Denver, CO, [4]Instituto Boliviano de Biología de Altura, La Paz, Bolivia. Email: Vaughn.Browne@uchsc.edu*

OBJECTIVE. Andeans (AND) with preeclampsia (PE) and Europeans (EUR) residing at high-altitude have reduced uterine artery (UA) blood flow, compared to normal AND (see other abstracts). We asked whether this resulted from blood flow redistribution in the common (CI) and external iliac (EI) arteries. METHODS. We studied 155 AND, 32 AND with PE (AndPE), and 38 EUR between 20–36 wk gestation. Using Doppler ultrasound, we calculated blood flow as mean velocity x vessel cross-sectional area. RESULTS. In EUR, blood flow was reduced 6 fold in UA, and ~20% in CI and EI arteries ($P<0.01$), due to smaller diameters, and slower mean velocities than in AND. Concurrently, the ratios of CI/UA and EI/UA diameter were ~30% higher ($P<0.01$), indicating that UA were disproportionately smaller in EUR. Consequently, the ratios of CI/UA and EI/UA blood flow were ~2.5 fold higher, indicating redistribution of blood flow to favor the lower body. In AndPE, CI and UA diameters were unchanged, but the EI were 8% larger ($P<0.01$), and the ratio of EI/UA diameter was 10% higher ($P<0.05$), compared to AND. While UA blood flow decreased 3-fold ($P<0.001$), due to lower mean velocity, CI and EI blood flow increased ~40% ($P<0.01$), due to higher mean arterial pressure, mean velocity, and larger EI diameter. Furthermore, the ratios of CI/UA and EI/UA blood flow were ~2.5 fold higher in AndPE, indicating that iliac blood flow increased to compensate for higher UA resistance, resulting in increased blood flow to the lower body. CONCLUSIONS. We speculate that compared to EUR, Andeans are genetically adapted to enlarge UA diameter, thereby redistributing blood flow to favor the UA. Defective remodeling of maternal end arteriolar vessels increases down-stream UA resistance and reduces mechanical distensibility in PE. (NIH-HL079647-01S1).

96. DIRECT EVIDENCE FOR INCREASED PULMONARY FREE RADICAL GENERATION IN AMS AND HAPE. Damian Bailey[1], Christoph Dehnert[2], Peter Bartsch[2], Heimo Mairbaeurl[2], Andrew Luks[3], Mariuz Gutowski[1], Elmar Menold[2], Vitaly Faoro[4], Christian Castell[2], Guido Schendler[2], Erik Swenson[3], Marc Berger[2], [5]. *[1]Department of Physiology, University of Glamorgan, UK, [2]Department of Internal Medicine VII, University of Heidelberg, Germany, [3]Department of Medicine, University of Washington, USA, [4]Department of Pathophysiology, University of Brussels, Belgium., [5]Department of Anesthesiology, University of Heidelberg, Germany. Email: dbailey[1]@glam.ac.uk*

The present study investigated the effects of high-altitude (HA) on the pulmonary exchange kinetics of the ascorbate free radical (A•-) and further examine if exchange is different in severe AMS and beginning HAPE. Thirty four subjects were examined at sea-level (SL) and within 3h (HA-1) and 20h (HA-2) following active ascent to 4559m. Resting blood samples were obtained from a central venous (superior vena cava) and radial arterial catheter for the direct detection of plasma A•- via EPR spectroscopy. Hemoglobin and hematocrit were measured to correct for plasma volume shifts. Pulmonary blood flow (PBF) was determined by a re-breathing technique and A•- exchange kinetics calculated via the Fick method [arterio-venous difference (a-vdiff) x (plasma) PBF]. AMS was diagnozed as a combined LL and ESQ-C score of ≥ 5 and ≥ 0.7 points respectively on HA-2. Chest radiography confirmed HAPE. Data were analyzed using a two factor repeated measures ANOVA and are expressed as mean ± SD. Fourteen subjects were diagnozed with AMS and 4 developed HAPE. Ten subjects remained healthy and 6 were excluded due to borderline AMS. An increase in the a-vdiff of A•- was observed at HA resulting in a net

outflow which was more pronounced in HAPE [SL: 9 ± 920 vs. HA-1: 1953 ± 311 vs. HA-2: 2388 ± 615 arbitrary units (AU)√Gauss (G)/min] and AMS (SL: 17 ± 637 vs. HA-1: 1580 ± 851 vs. HA-2: 846 ± 378 AU√G/min) compared to healthy controls (SL: 145 ± 1007 vs. HA-1: 490 ± 1183 vs. HA-2: 146 ± 683 AU√G/min, $P < 0.05$). These findings provide the first direct evidence for a net outflow or release of free radicals across the lungs at HA which may have implications for the pathophysiology of AMS and HAPE. Funding: SRIF-III.

97. TEMPORARY VISION LOSS AT ALTITUDE DUE TO INTERMITTANT ANTERIOR CHAMBER ANGLE CLOSURE. RS Davidson[1], MY Kahook[1], RC Roach[2], B Honigman[3]. *[1]Assistant Professor of Ophthalmology, Rocky Mountain Lions Eye Institute, University of Colorado School of Medicine, [2]Associate Professor of Surgery, Research Director, Altitude Research Center, University of Colorado School of Medicine, [3]Professor of Surgery, Director, Altitude Research Center, University of Colorado School of Medicine. Email: benjamin.honigman@uchsc.edu*

Purpose: To describe a case of intermittent anterior chamber angle closure occurring on 3 occasions in the left eye of a 56 year-old man while hiking at night at altitudes above 12, 000 feet, and resolving with descent over 2-3 hours. Methods: The patient was examined by two ophthalmologists at the Rocky Mountain Lions Eye Institute (RMLEI) and then taken to the Altitude Research Center (ARC) for decompression to 14, 000 feet (PB = 430 mmHg) in a hypobaric chamber for eighty minutes. During 40 minutes of "ascent" he exercised on a cycle ergometer to simulate hiking. His intraocular pressures (IOP) and visual acuity were monitored throughout. He returned to RMLEI for additional eye exams while breathing 12% oxygen. Results: Prior to ascent, examination showed "occludable" anterior chamber angles in both eyes. During the evaluation in the altitude chamber, he had some fluctuation in IOP and visual acuity in both eyes, however the exact symptoms could not be reproduced for a sustained period of time. Visual acuity upon presentation was 20/20 at near in both eyes. It decreased to 20/400 in the right eye and 20/30 in the left eye after 50 minutes at 14, 000 feet and IOP ranged from 13-16mm Hg in each eye. Pachymetry measurements and retina evaluation were within normal limits. Conclusions: While we were not able to completely reproduce the patient's symptoms, he did experience slight visual disturbances and IOP fluctuations possibly connected to his occludable angles. Intermittent angle closure can result in transient rises in IOP and corneal edema which lead to vision loss. We hypothesize that the combination of hiking at night (which results in pupillary dilation) and the hypoxia induced by the high altitude led to these findings. The authors have no financial interest in the subject matter presented.

98. EPIDEMIOLOGY OF TREKKERS ATTEMPTING MT KILIMANJARO. Andrew Davies[1], Suzy Stokes[1], Adam Whitehead[1], Ian Tyrrell-Marsh[1], Mark Earl[1], Hannah Frost[1], Nicholas Kalson[1], Nicholas Truman[1], Jon Naylor[2]. *[1]Manchester Altitude Research Society, University of Manchester, [2]Peterborough District Hospital. Email: andrewjohndavies@hotmail.com*

Mt Kilimanjaro (5895m) is the highest mountain in Africa. Thirty thousand people attempt to climb it each year, of which many fail to reach the summit and many suffer acute mountain sickness (AMS). Our study followed the physiological and physical progress of 183 tourists as they attempted to ascend the Marangu route of Mt Kilimanjaro during a 17 day period in August 2005. This route has a fixed ascent profile, with an entrance gate at 1980m, huts at 2700m, 3700m and 4700m. All trekkers were required to stay at each of the huts during ascent, with the choice of spending extra nights at 3700m to acclimatise. All of our subjects attempted the summit after one night at 4700m before returning to 3700m for their last night on the mountain. Here we present the epidemiological facts, including a description of the trekkers who attempt this route, their

success rate, the prevalence of AMS and other relevant information. Of the 183 complete sets of data, only 61.7% achieved the summit, 13.7% achieved Gilman's Peak at 5600m, 8.2% did not leave the final hut to make a summit bid, and the remainder stopped at various intermediate altitudes. 59.8% were males, average age was 34 (SD 12.2, range 11 – 70), and average BMI was 23 (SD 3.2, 17.2 – 33.2). 36% were suffering from AMS before attempting the summit, and 78% experienced AMS on their summit day (Lake Louise Score (LLS) ≥ 4. One score of 21 was noted, with two LLS of 17 and 2 of 16. Some co-morbidities were noted, including 3.9% with previously diagnosed hypertension, 5.8% asthmatics, 3.9% diabetics (including a group of Canadian type 1 diabetics), 3.8% with recent (previous week) upper respiratory tract infection, and 6.8% with diarrhoea and vomiting.

99. NO CHANGE OF LUNG VOLUMES AND COMPLIANCE MEASURED BY BODY PLETHYSMOGRAPHY IN AMS AT 4559 M. Christoph Dehnert[1], Andrew Luks[2], Guigo Schendler[1], Elmar Menold[1], Marc M. Berger[3], Christian Castell[1], Vitalie Faoro[4], Heimo Mairbäurl[1], Damian M. Bailey[5], Erik R. Swenson[2], Peter Bärtsch[1] *[1]University Hospital Heidelberg, Internal Medicine VII, Sports Medicine, Heidelberg, Germany[2]VA Puget Sound Health Care System, University of Washington, Division of Pulmonary and Critical Care Medicine, Seattle, USA[3]University Hospital Heidelberg, Department of Anaesthesiology, Heidelberg, Germany[4]Department of Pathophysiology, University of Brussels, Belgium[5]University of Glamorgan, Department of Physiology, Pontypridd, United Kingdom. Email: christoph.dehnert@med.uni-heidelberg.de*

Previous studies of pulmonary function at high altitude have shown a decrease in lung volumes which has been attributed to mild interstitial pulmonary edema and partly associated with acute mountain sickness (AMS). To examine whether these changes in lung volume can be attributed to subclinical edema or result from other factors, we conducted an extensive pulmonary function testing (PFT) program including body plethysmography compliance measurements, maximum voluntary ventilation (MVV) and breathing forces in 34 healthy subjects at an altitude of 100 m (LA) and 20h after rapid ascent to 4559m (HA). AMS was defined as the combination of a Lake-Louise Score ≥5 AND an AMS-C-score ≥0.70. 6 subjects with uncertain diagnosis regarding AMS and 4 subjects with HAPE were excluded from the presented analysis. There were no statistically significant differences in total lung capacity, vital capacity, FEV1 and static compliance between LA and HA. At HA, no differences were found in these parameters between individuals with and without AMS. Vital capacity measurements at HA were consistently 5-15% higher than those reported in prior studies. MVV significantly increased from 169±28 at LA to 204±38 at HA in subjects with AMS and from 159±25 at LA to 189±31 at HA in individuals without AMS (2-Way-ANOVA, $p < 0.05$). Maximum inspiratory pressures decreased from 11.7±2.6 at LA to 11.0±2.3 at HA when all subjects were analyzed together. The lack of observed changes in lung volumes and static compliance argue against the presence of subclinical pulmonary edema in individuals with and without AMS following rapid ascent to high altitude. The fact that vital capacities, expressed as %-predicted, were 5-15% higher in our study than in previous studies suggests that a lower degree of participant effort may account for results observed in previous studies.

100. SLEEP QUALITY AND NOCTURNAL DESATURATION DURING AN EXTENDED STAY AT HIGH ALTITUDE. Gerald Dubowitz[1], Allison Mulcahy[2], Hale Hansen[3]. *[1]Department of Anesthesia, University of California San Francisco, [2] Alameda County Emergency Medical Center, Highland Hospital, Oakland CA, [3] Department of Medicine, University of Washington School of Medicine. Email: dubowitz@anesthesia.ucsf.edu*

Background: Many studies have reported disturbed sleep in lowlanders ascending to high altitude. While most have studied one or two nights, few have looked at extended stays. We hypothesized that sleep disturbance decreases during a longer sojourn at altitude, correlated with an improvement in desaturation events. We therefore set out to record sleep patterns during 5-9 days at 3801m and 4250m. Subjects: 10 healthy lowlanders ascending to 3801m or 4250m from sea level. Methods: Pulse oximetry was measured every 2 seconds during sleep using a wrist pulse-oximeter. Subjects were asked to describe sleep quality using a subjective sleep score. Results: Significant desaturation events (the number of dips 4% below the mean saturation and lasting more than 10 seconds) ranged from 4-111events/hr and did not significantly change from day 1 to 9, although sleep quality and Lake Louise acute mountain sickness (AMS) score markedly improved after night 2. A trend towards fewer desaturation events with increasing time at altitude was present, but was not significant. Desaturation events were still noted after 30 days at 4250m (n=1). The number of events/hr was greater at 4250m compared with 3801m. There was no significant change in mean overnight saturation (range 81-86%) with increasing time at altitude. No correlation was found between the number of events, AMS score or sleep quality. Conclusions: We conclude that desaturation events do not normalize (acclimatize) after 9 days at altitude and may take significantly longer to return to sea-level values during a continuous stay at altitude. Subjective sleep quality is not directly related to desaturation events; in spite of persistent desaturation events, improved sleep was reported. This may be due to other factors contributing to perceived sleep quality or to more subtle changes or events not detected in this study.

101. DETERMINANTS OF ACUTE MOUNTAIN SICKNESS AND SUMMIT SUCCESS ON MOUNT KILIMANJARO. Andrew Davies[1], Suzy Stokes[1], Nicholas Kalson[1], Hannah Frost[1], Mark Earl[1], Ian Tyrrell-Marsh[1], Adam Whitehead[1], Nicholas Truman[1], Jon Naylor[2]. *[1]Manchester Altitude Research Society, University of Manchester, [2]Peterborough District Hospital. Email: andrewjohndavies@hotmail.com*

We examined basic physiological factors in 183 subjects attempting the summit of Mt Kilimanjaro (5895m) and investigated whether these correlated with both Acute Mountain Sickness (AMS) and summit success. Our study assessed 183 trekkers attempting the Marangu route of Mt Kilimanjaro. This route has a fixed ascent profile, where trekkers must spend one or two night in huts at 2700m, 3700m, and 4700m before attempting the summit. Our teams were based at all three huts where we took physiological readings from every subject each night. These included blood pressure, heart rate, respiratory rate (RR), and arterial oxygen saturations (SaO_2). We then compared these values to whether the subjects summited successfully and to the severity of their AMS using the Lake Louise Scoresheet (LLS). The data was analysed to see if it was possible to predict both the summit success and presence of AMS using physiological factors from lower altitudes. Several physiological values were found to have significant relationships. Regarding whether or not a subject achieved the summit, we found statistical significance, and therefore some predictive value, with SaO_2 on arrival at 3700m ($p<0.01$, OR: 1.536) and at 4700m ($p<0.01$, OR: 1.790), and mean arterial pressure (MAP) ($p=0.042$, OR: 1.787) and RR ($p<0.01$, OR: 1.754) on arrival at 4700m. In terms of predictability of AMS, we found that SaO_2 (3700m $p=0.019$, OR: 0.269. 4700m $p<0.01$, OR: 0.284) and MAP (3700m $p=0.015$, OR: 0.262. 4700m $p<0.027$, OR: 0.271) at both 3700m and 4700m correlated well with presence of AMS upon arrival at 4700m, whilst SaO_2 at 4700m ($p<0.01$, OR: 2.392) was also significant regarding

presence of AMS on summit day. Our data provides possible physiological predictors for individuals who are attempting to reach the summit of Mt Kilimanjaro.

102. SYSTEMIC NITRIC OXIDE BIO-AVAILABILITY IS NOT IMPLICATED IN THE PATHOPHYSIOLOGY OF AMS. KA Evans[1], PP James[2], PN Ainslie[3], L Fall[1], P Martins[1], E Kewley[1], DM Bailey[1]. *[1]Hypoxia Research Unit, Department of Physiology, University of Glamorgan, UK, [2]Wales Heart Research Institute, University of Wales College of Medicine, UK, [3]Department of Physiology, University of Otago, New Zealand. Email: kaevans@glam.ac.uk*

Acute exposure to inspiratory hypoxia is associated with increased free radical generation which may have implications for nitric oxide (NO) bio-availability, neurovascular function and susceptibility to acute mountain sickness (AMS). The present study tested the hypothesis that acute exposure would decrease the systemic concentration of NO and that this response would be more marked in subjects who developed AMS (AMS+) compared to their healthier counterparts (AMS-). Following ethical approval and written informed consent, venous samples were obtained from 18 males without a prior history of AMS in normoxia (21% O_2-N) and following 6h passive exposure to normobaric hypoxia (12% O_2-H). The plasma (200µL sample injection) and red blood cell (100µL injection) concentrations of total nitric oxide (NOx) were assayed using a modified ozone-based chemiluminescence technique and were not corrected for plasma volume shifts. Clinical AMS (moderate to severe) was diagnosed if a subject presented with a cumulative Lake Louise score (self assessment + clinical score) of ≥5 points and Environmental Symptoms Questionnaire Cerebral Symptoms score ≥0.7 points. Data were not normally distributed and subsequently analyzed using a Mann-Whitney U Test and are expressed as mean ± SD. Nine subjects were diagnosed with AMS (50% of group). No within or between group differences were observed during either N or H in the plasma (N-AMS+: 486.8 ± 123.8 vs. N-AMS-: 515.8 ± 179.4 nmol/L, $P > 0.05$ and H-AMS+: 542.4 ± 141.2 vs. H-AMS-: 447.6 ± 87.0 nmol/L, $P > 0.05$) or RBC (N-AMS+: 118.1 ± 91.0 vs. N-AMS-: 180.7 ± 156.5 nmol/L, $P > 0.05$ and H-AMS+: 168.8 ± 72.2 vs. H-AMS-: 173.3 ± 71.5 nmol/L, $P > 0.05$) concentration of NOx. These findings suggest that "systemic" NO bio-availability is not implicated in the pathophysiology of AMS.

103. CHEMORECEPTOR RENIN-ANGIOTENSIN SYSTEM ACTIVITY AND THE VENTILATORY RESPONSE TO ACUTE HYPOXIA. Forth, RJ[1,2], Humphries, SE[2], Prisk, GK[3], West, JB[3]. *[1]Institute of Health and Human Performance, Royal Free & University College London Medical School, London, UK, [2]Centre for Cardiovascular Genetics, British Heart Foundation Laboratories, Royal Free & University College London Medical School, London, UK, [3]Division of Physiology, Dept. of Medicine, University of California, San Diego, La Jolla, CA, USA. Email: r.forth@ucl.ac.uk*

Animal models suggest the existence of a carotid body chemoreceptor renin-angiotensin system (RAS) which influences type I glomus cell activity. A human angiotensin converting enzyme (ACE) polymorphism involving a 287 base-pair insertion (I) or deletion (D) in intron 16 is associated with lower and higher tissue ACE activity respectively. The ACE genotype is associated with high-altitude performance. This could be mediated through alterations in the hypoxic ventilatory response (HVR), as presence of the ACE I allele is associated with better sustained SaO_2 during rapid ascent to 5300m. Isocapnoeic HVR (iHVR) was measured using a rebreather technique on 133 healthy Caucasian male volunteers (mean age ± SD: 29.9 ± 5.32 years), who were later genotyped for the ACE I/D polymorphism and also for the RAS downstream receptor polymorphisms AT1R1166A>C, the X chromosome-located AT2R1675A>G and BK2R +9/-9, each of which is associated with differing activity/expression of the corresponding receptor. Subjects' baseline characteristics and fitness levels including VO_2max and anaerobic threshold

were within the normal ranges for untrained individuals and were independent of genotype. Subjects with the ACE I allele had a significantly more responsive mean iHVR than those who did not (P = 0.013; mean ± SD: I allele present -1.57 ± 0.85 Lmin-1SaO_2-1, no I allele -1.17 ± 0.76 Lmin-1SaO_2-1). Heterozygosity of AT1R1166A>C was associated with a more responsive mean iHVR than homozygosity at this locus (P = 0.046; mean ± SD: AC genotype -1.61 ± 0.97 Lmin-1SaO_2-1, DD or CC genotype -1.31 ± 0.71 Lmin-1SaO_2-1). The AT2R1675A>G and the BK2R +9/-9 polymorphisms did not show any significant association with iHVR. This is the first study to demonstrate that the response to hypoxia as an isolated stimulus is significantly associated with the ACE and AT1R polymorphisms. This study was funding by the Colt Foundation, Great Ormond Street Hospital and the Portex Endowment.

104. VENTILATORY AND CARDIOVASCULAR RESPONSES TO REPEATED SHORT TERM HYPOXIC EXERCISE. Markus Flatz[1], Mark Olfert[2], Susan Hopkins[2]. *[1]Department of Sport Science, University of Innsbruck, Austria[2]Department of Medicine, University of California at San Diego, La Jolla, California, US. Email: markus.flatz@uibk.ac.at*

Intermittent hypoxic exercise training is suggested to increase the hypoxic ventilatory response and improve aerobic performance. To test the effects of repeated hypoxic exercise on cardiorespiratory responses, we conducted daily exercise tests using cycle ergometry consisting of a warm up, 6 minutes of steady-state exercise at 65% of maximal followed by 30Watt/2min increments to exhaustion ($V.O_2$max) in hypoxia (12.5% O_2) in 6 active subjects (age=25±4 years, normoxic $V.O_2$max=49±9ml/kg/min) with normal pulmonary function. After preliminary testing to establish baseline normoxic and hypoxic $V.O_2$max values, data including ventilation, oxygen consumption, and cardiac output (open circuit acetylene uptake) were collected at rest and during exercise for 4 consecutive days (H1 to H4). At rest and during maximal exercise heartrate, cardiac output, oxygen consumption and minute ventilation (V. E) were unchanged, as was $V.O_2$max(p=0.3). During steady-state exercise cardiac output and $V.O_2$ were also unchanged, however V. E progressively increased 12% by H4 (means±SD=58±11 l/min BTPS H1, 58±11 H2, 62±14 H3, 65±13 H4, p=0.03). Since steady-state $V.O_2$ was unchanged, V. E/$V.O_2$ and V. E/power relationships also increased (p=0.004 and 0.031, respectively). Alveolar ventilation (V. A) tended to increase (p=0.09) however oxygen saturation (SpO_2) was unchanged (71±7% H1, 71±10 H2, 73±6 H3, 74±7 H4, p=0.6). These data suggest that four repeated daily bouts of short-term hypoxic exercise is sufficient to augment the ventilatory response to steady state hypoxic exercise, in the absence of significant changes at rest. These changes may be more pronounced and potentially affect performance during endurance exercise of longer duration. Support:Tyrolean Research Foundation (GZ:UNI-0404/216), Department of International Relationships of the University of Innsbruck, Austria (GZ:36001/28-05), AHA 0540002N, Parker B. Francis Foundation.

105. CLIMBING SPECIFIC FINGER ENDURANCE IN NORMOXIA AND HYPOXIA. Stanley Grant[1], Jamie Reid[1], Dave MacLeod[1], John Bradley[2], Ronald Baxendale[1], Neil Innes[1], Mhairi Stewart[1], Alice Moir[1], Tom Aitchison[3]. *[1]Institute of Biomedical and Life Sciences, University of Glasgow, [2]Scottish Institute of Sport, [3]Statistics Department, University of Glasgow. Email: S.Grant@bio.gla.ac.uk*

Finger endurance is an especially important attribute in rock climbing. The aim of this study was to compare the effect of hypoxia on endurance performance in a climbing specific task in climbers and non-climbers. Finger climbing specific endurance of 10 climbers (27.0 ± 4 years) with an 'on-sight' ability of at least 7a on the French scale and 12 non-climbers (21.3 ± 2 years) was compared. Maximum voluntary contraction (MVC) trials and isometric endurance tests (in-

termittent contractions of 10 seconds, with 3 second rests) were performed (using four fingers of the right hand on a climbing specific apparatus) at 40% MVC in normoxia and hypoxia until volitional exhaustion. Surface EMG was recorded in the finger flexors throughout each of the endurance tests. Significance level was taken at $P<0.05$. Climbers had a higher MVC (climbers – 423 ± 56 N; non-climbers - 298 ± 42 N). There were no differences for endurance time for both groups in normoxia and hypoxia (Normoxia: climbers - 223.2 ± 102 s; non-climbers - 164.9 ± 57 s; Hypoxia: climbers - 155.7 +53 s; non-climbers - 131.5 + 51.5 s). Force-time (f-t) integral per kg body mass (used as a measure of climbing specific endurance and calculated as follows - [40%*MVC (in Newtons) * total endurance time (s)] / body mass [kg]), was higher in climbers (Normoxia: climbers - 537.5 ± 247 Ns/kg; non-climbers - 265.7 ± 110 Ns/kg; Hypoxia: climbers - 375.0 ± 143 Ns/kg; non-climbers - 222.0 ± 103 Ns/kg). The superior f-t integral scores of the climbers cannot be attributed to differences in the median frequency of the EMG signal.

106. HYPOXIA REGULATES MITOCHONDRIAL PROTEIN EXPRESSION OF SKELETAL MUSCLE IN WISTAR RATS: A PROTEOMIC STUDY. Wenxiang Gao, Jian Chen, Yuqi Gao, Jian Huang, Mingchun Cai. *Institute of High Altitude Medicine, Third Military Medical University, Chongqing 400038, China.*

Objective: To detect regional differences of protein expression levels in gastrocnemius mitochondrial fractions of Wistar rats exposed to hypobaric hypoxia and the control by use of proteomic methods. Methods: Adult male Wistar rats were randomized into hypoxic (4500 m, 30 d) group and the normoxic control group (sea level). Bilateral gastrocnemius muscles were collected and mitochondria were extracted and purified. Mitochondrial oxygen consumption was measured with Clark oxygen electrode, mitochondrial transmembrane potential was detected with Rhodamine 123 as a fluoresce probe. Two dimensional electrophoresis and PDQuest were applied and the peptide mass fingerprinting of differential proteins was analyzed with matrix assisted laser desorption/ionization mass spectrometry analysis (MALDI-TOF MS). For two of the spots, the expression patterns were confirmed by Western blotting analysis. Results: Using 2-DE and MALDI-TOF MS, we identified 8 mitochondrial protein spots that were differentially expressed in the hypoxic group compared with the normoxic control. These proteins included Chain A of F1-ATPase, voltage dependent anion channel 1, hydroxyacyl Coenzyme A dehydrogenase alpha subunit, mitochondrial F1 complex gamma subunit, androgen-regulated protein and tripartite motif protein 50. Interestingly, most of these proteins are associated with the mitochondrial respiratory chain and energy metabolism. Conclusion: With successful use of multiple proteomic analysis techniques, we demonstrates that 30 d hypoxia exposure may have effect on the Krebs cycle and lipid metabolism, decrease the stability of mitochondrial membrane and affect the mitochondrial electron transport chain. Acknowledgement: This work was supported by National Natural Science Foundation of China (NSFC) No.30300123 and No.30393131. (*Corresponding: gaoy66@yahoo.com).

107. MITOCHONDRIAL MECHANISMS OF HIGH ALTITUDE ADAPTATION OF TIBETAN. Yuqi Gao[1], Xiuxin Zhao[1], Wenxiang Gao[1], Lang Suo[2], Jian Chen[1]. *[1]Institute of High Altitude Medicine, Third Military Medical University, Chongqing [4]000[3]8, China., [2]Department of gynaecology and obstetrics, the first People's Hospital of Tibet autonomous region, Lhasa 8[5]0000, China. Email: gyq@mail.tmmu.com.cn*

Objective: To explore the mitochondrial mechanisms of high altitude adaptation of native Tibetan in Tibetan Plateau. Methods: The subjects were Tibetan and Han normal pregnant women. After delivery, infant body weight and length as well as placenta weight and volume were determined, and placental ratio was calculated. The placenta samples were collected and mitochondrial respiratory state III (ST3) and IV (ST4), respiratory control ratio as well as activity of

mitochondrial oxidative phosphorylation complex I, II and IV were measured with Clark electrode. F0F1-ATP synthase activity and ATP production were measured with inorganic phosphorus measurement. ADP/ATP carrier activity was measured with 3H-ADP incorporation. Results: Placenta weight and volume as well as infant body weight and length were significantly higher, while placental ratio was significant lower, in Tibetan than in Han. ST3, respiratory control ratio and oxidative phosphorylation rate of placenta mitochondria were significantly higher in Tibetan than in Han, while ST4 showed no significant difference between them. Activity of mitochondrial oxidative phosphorylation complex I and II, F0F1-ATP synthase and ADP/ATP carrier, as well as ATP production was significant higher in Tibetan than in Han. No marked difference was found in complex IV activity between Tibetan and Han. Conclusion: Mitochondrial oxidative phosphorylation activity is significantly higher in native Tibetan than immigrant Han, indicating that Tibetans are able to utilize more oxygen to generate ATP under hypoxic conditions in high altitude. This may be an important mechanism for Tibetan high altitude adaptation. Acknowledgements: This work was supported by NSFC No.30300123 and No.30393131.

108. SKELETAL MUSCLE IN PATIENTS WITH CHRONIC OBSTRUCTIVE LUNG DISEASE: PROTEIN AND ISOFORM ABUNDANCE AND MAXIMAL CATALYTIC ACTIVITY OF THE NA+-K+ATPASE. H. Green[1], T. Duhamel[1], C. D'Arsigny[2], D. O'Donnell[2], I. McBride[2], J. Ouyang[1]. *[1]University of Waterloo, Waterloo, Ontario, Canada, [2]Respiratory Investigation Unit, Kingston General Hospital, Kingston, Ontario, Canada. Email: green@healthy.uwaterloo.ca*

Introduction: The aim of this study was to investigate the hypothesis that protein abundance, isoform content and maximal catalytic activity of the Na+-K+-ATPase would be compromised in skeletal muscle of patients with moderate to severe chronic obstructive pulmonary disease. Methods: Tissue samples were obtained from the vastus lateralis muscle of 8 patients with chronic obstructive pulmonary disease (COPD, age 66.6±3.4 yr; body mass 76.2±5.6 kg; FEV1.0 =0.80±0.06, l/min) and 10 healthy-matched controls (CON, age 67.5±2.4 kg; body mass 74.1±5.5 kg; FEV1.0 = 2.43±0.19, l/min) and assessed for maximal catalytic of the enzyme (Vmax) using the K+-stimulated 3-O-methylfluorescein phosphatase(3-O-MFPase) assay, enzyme abundance, measured by [3H] ouabain and isoform content of both the α (α1, α2, α3) and β (β1, β2, β3) subunits using Western blot techniques. Resting paO_2 (mm Hg) was 83.8±2.2 and 61.4±2.3 in CON and COPD, respectively. Results: A 19.4% lower ($P<0.05$) Vmax was observed in COPD compared to CON (90.7±6.7 vs 73.1±4.7 nmol.mg protein-1.h-1. No differences between groups were observed for Na+-K+-ATPase concentration (259±15 vs 243±17 pmol.g wet wt-1). For the α isoforms, α1 was depressed ($P<0.05$) by 28% and α2 was increased by 12% ($p=0.06$) in COPD compared to CON while no differences existed between groups for α3. No differences in β subunit expression (β1, β2, β3) were observed. Conclusion: As hypothesized, moderate to severe COPD is accompanied by disturbances in Vmax of the enzyme which occurs in the absence of changes in total protein abundance. The reduction in maximal Ca2+-ATPase activity could be mediated either by a shift in α isoform expression (α1, α2), alterations in intrinsic regulation or structural changes in the enzyme. The changes observed in maximal catalytic activity may have important consequences to membrane excitability and fatiguability typically compromised in COPD. Acknowledgements Supported by the Department of Medicine Research (Queen's University).

109. CLINICAL APPLICATION OF A STANDARDIZED STIMULUS TO ASSESS CEREBROVASCULAR REACTIVITY USING BOLD MRI. Jay Han[1,2], Marat Slessarev[2], Danny Mandell[3], Julien Poublanc[3], Adrian Crawley[3], Alexandra Mardimae[1,2], Joseph A. Fisher[1,2], David J. Mikulis[3]. *[1]Department of Physiology, University of Toronto, [2]Department of Anesthesia, University Health Network, [3]Department of Medical Imaging, University Health Network. Email: Jay.Han@uhn.on.ca*

Introduction: Cerebrovascular Reactivity (CVR) is defined as a change in cerebral blood flow (quantified as a change in BOLD MRI signal) for a given change in end-tidal PCO_2; $PETCO_2$). The magnitude of CVR is indicative of the extent of flow reserve in a vascular territory and is strongly related to risk of stroke. An absolute measurement of global flow reserve requires a standard $PETCO_2$ stimulus. We present 3 cases illustrating the clinical advantages of measuring CVR with a reproducible stimulus. Methods: We present CVR results from one healthy subject (H), one asymptomatic patient with severe bilateral carotid stenosis (A), and one symptomatic patient with left middle cerebral artery (MCA) stenosis (S). A standardized hypercapnic stimulus consisting of two cycles of $PETCO_2$ cycling between 40 ± 1.32mmHg and 50 ± 1.92mmHg while maintaining iso-oxia (end-tidal PO_2 100 ± 5.02mmHg) was applied to each patient while BOLD MR signal was acquired. CVR was then calculated and mapped voxel by voxel throughout the brain. Results: The CVR for A was similar in magnitude and distribution to that of H. S however had a reduced CVR in left MCA territory, corresponding to the vascular territory at risk and the patient's symptoms. S also had a greater CVR in the vascular area of the right MCA territory. Difference in CVR normalized for area (BOLD signal/mm $PETCO_2$ X total number of voxels in the vascular territory) in left MCA territory between S and average of A and H was - 11312.3 (arbitrary units) while the difference in right MCA was + 27043.80 (arbitrary units). Conclusion: This illustrates how a standardized CVR test will allow us to quantitatively assess the impact of severe arterial compromise on the flow reserve of the affected vascular territory and identify any compensatory changes in the flow reserve of the contra-lateral vascular territory.

110. HYPERCARBIA DURING REPEATED RADIOTHERAPY MAY REDUCE TUMOR RADIOSENSITIVITY: EARLY OBSERVATIONS. Jay Han[1,2], Marat Slessarev[1], [2], Daniel Mandell[3], Julien Poublanc[3], Alexandra Mardimae[1,2], Eitan Prisman[2], Barbara-Ann Millar[4,5], Normand Laperriere[4,5], Joseph A. Fisher[1,2], David J. Mikulis[3], Cynthia Menard[4], [5]. *[1]Department of Physiology, University of Toronto, [2]Department of Anesthesia, University Health Network, [3]Department of Medical Imaging, University Health Network, [4]Radiation Medicine Program, University Health Network, [5]Department of Radiation Oncology, University of Toronto. Email: Jay.han@uhn.on.ca*

Introduction: Brain tumors contain aberrant neo-vascular networks and hypoxic regions, which are resistant to radiation. Carbogen (either 2 - 5% CO_2 in O_2) is sometimes administered as an adjuvant to radiotherapy in an attempt to increase tumor blood flow and restore oxygenation and radiosensitivity. However, the effect of O_2 and CO_2 on the distribution of brain blood flow in the course of radiotherapy is unknown. We studied the Cerebro-Vascular Reactivities (CVR) in two patients with brain tumors before, and during the course of 3D conformal radiotherapy. Methods: The reproducible provocative stimulus consisted of changes in end-tidal PCO_2 (Pet-CO_2) between 35 and 45 mmHg at normoxia (PO_2 100 mmHg) and hyperoxia (PO_2 400 mmHg). MRI BOLD signal was used as an indicator of cerebral blood flow. CVR, quantified as changes in BOLD MR signal per mmHg change in $PetCO_2$, was determined prior to, and after administration of radiotherapy targeted at the tumor and a margin of surrounding brain. Results: Prior to initiation of radiotherapy, CVR in the tumor matched that of normal brain with small regions of paradoxical reactivity. Five weeks after initiation radiotherapy, the high-dose region—tumor and surrounding edematous brain—showed enlarged areas of paradoxical reactivity compared

to that in the low-dose region of the brain. BOLD signal changes in response to changes in PO_2 were too subtle to draw definitive conclusions. Conclusion: Radiation therapy appears to abolish the regional vascular reactivity to CO_2, resulting in paradoxical redistribution of blood flow from high-dose to low-dose regions of the brain in response to hypercarbia. Thus, carbogen may reduce tumor blood flow—and thereby radiosensititvity—during the course of radiotherapy.

111. ROLE OF EPO AND NMDA RECEPTORS IN VENTILATORY ACCLIMATIZATION TO HYPOXIA IN A MODEL OF ANEMIC TRANSGENIC MICE. Raja El Hasnaoui, Thierry Launay, Aurélien Pichon, Patricia Quidu, Alain Duvallet, Jean-Paul Richalet, Fabrice Favret. *Université Paris 13, EA2363 "Réponses cellulaires et fonctionnelles à l'hypoxie". Bobigny, France. Email: rajaelhasnaoui@hotmail.com*

Both polycythemia and hyperventilation are considered as main factors to adapt to low oxygen tension. It has been previously shown that transgenic anemic mice (Epo-TAgh) adapt to hypoxia partly through an increased acute Hypoxic Ventilatory Response (AHVR) which contributes to an improved ventilatory acclimatization to hypoxia (VAH). It has been suggested that hypoxia increased the responses of respiratory centers to afferent inputs from carotid body chemoreceptors and that this process involved NMDA-Receptors (NMDA-R) mediated mechanisms. In addition, recent studies showed that Epo via Epo-Receptors (EPO-R) controlled ventilation in hypoxia at the central and peripheral levels and that endogenous Epo system was affected following blockade of NMDA-R function. Thus, we hypothesized that NMDA-R and EPO-R could be involved in the regulation of the ventilation in Epo-TAgh and in Wild type mice in AHVR and in VAH. Two groups of Epo-Tagh and 2 groups of Wild-Type mice were exposed during 2 weeks either to hypoxia (~4500 meters) or to normoxia. After exposure, the medulla were removed to measure gene expression of NMDA-R and EPOR by real time RT-PCR. We showed first that, in normoxia, anemia resulted in a marked increase in EPOR (+284%) and NMDA-R1 (+233%) gene expression in the medulla. Chronic hypoxia led to a rise in EPOR (+143%) and NMDA-R1 (+161%) mRNA in Wild Type mice. In contrast, neither acute nor chronic hypoxia had an effect on EPOR or NMDA-R mRNA of Epo-TAgh mice. In conclusion, our results suggest that EPOR and NMDA-R could participate in AHVR and probably to VAH of Wild Type mice. Nevertheless, while it is possible that these receptors participate in AHVR of Epo-TAgh mice, their role in the VAH remain to be determined.

112. INTERNATIONAL HAPE REGISTRY SCORE PROPOSED. N. Stuart Harris[1], Peter Bartsch[2], Buddha Basnyat[3], Marco Maggiorini[4], James Milledge[5], Susan Niermeyer[6]. *[1]Department of Emergency Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, [2]Universitätsklinikum Heidelberg, Medizinische Klinik (Krehl-Klinik), Innere Medizin VII: Sportmedizin, Heidelberg, Germany, [3]Nepal International Clinic, Katmandu, Nepal, [4]Department of Internal Medicine, University Hospital Zürich, Zürich, Switzerland., [5]Hon. Professor University College, London., [6]Neonatology, The Childrens Hospital, Denver, Colorado. Email: nsharris@partners.org*

The International HAPE Registry's Steering Committee has suggested the following criteria, known as the International HAPE Registry Score, to quantify the likelihood of a HAPE diagnosis entered into the Registry. The score is calculated by simply adding the number of points assigned for each positive criterion. HAPE Registry Scores may range from 0 to 21 points. Current symptoms: Dyspnea or orthopnea at rest (2 points); Incapacitating dyspnea with exercise (2). Current signs or physical findings: Pulse oximetry <70% at rest (except when above 6000 m) (2); Reddish/brown or frothy sputum (2); Rales: 1 point per side affected. If unilateral, 1 point. If bilateral, 2 points. (1 or 2). Historic factors: Physician diagnosed prior history of HAPE (2); Alti-

itude of onset of symptoms: >3000 m (1); Average rate of ascent on the two preceding days >1000 m/day (1); Heavy physical activity in the two days immediately prior to onset of symptoms: (e.g. running, climbing, mountaineering) (1). Additional diagnostic testing data: Radiographic score of at least two in any lung field (2) OR Bilateral transthoracic ultrasonography reveals a total of 15 or more comet-tail signs (2); Echocardiogram within 72 hours of symptoms showing pressure gradient across the tricuspid valve > 50 mmHg and normal left ventricular function (EF > 50%, no LV wall motion abnormalities. (2); ECG finding of Right axis deviation or signs of right ventricular overload (definition to be decided) or P pulmonale (as defined by the presence of a P wave 2.5 millimeters or greater in height in leads II, III, or aVF) (1). Interventions: Rapid improvement (< 24 hours) of gas exchange or clinical performance after descent of > 3200 ft/ 1000 m, or administration of oxygen, any calcium channel blocker, phosphodiesterase-5-inhibitor or any other vasodilator that affects the pulmonary circulation (2).

113. ACCURATE INDUCTION AND MEASUREMENT OF ALVEOLAR PCO_2. Shoji Ito[2], Alexandra Mardimae[1,2], Ludwik Fedorko[2], Rita Katznelson[2], Leonid Minkovich[2], Cathie Kessler[2], Jay Han[1, 2], Marat Slessarev[1,2], James Duffin[1], Joseph Fisher[1,2]. *[1]Department of Physiology, University of Toronto, [2]Department of Anaesthesia, University Health Network. Email: sho2ito@yahoo.co.jp*

Background: Alveolar PCO_2 ($PACO_2$) calculated from reconstructed alveolar CO_2 concentrations or from regression equations does not accurately predict $PaCO_2$ (SD 3-5 mmHg). We reasoned that when breathing via a sequential gas delivery breathing circuit (SGD) with rebreathing, if minute ventilation exceeded the fresh gas flow (FGF), the FGF becomes equal to alveolar ventilation and for a given CO_2 production (VCO_2), $PETCO_2$ should equal $PaCO_2$. Methods: VCO_2 measurements used to target $PETCO_2$ were made in 3 healthy middle aged subjects in whom indwelling arterial catheters were placed. Subjects breathed via an SGD and were then asked to synchronize their breathing frequency (RR) to 12, 18 and 24 min-1. At each RR, FGF composition was adjusted a) to three target $PACO_2$ ($tPACO_2$) levels between 30 and 50 mmHg at iso-oxia ($PETO_2$ = 100 mmHg) and b) to $tPACO_2$ of 40 mmHg while changing $PETO_2$ to 70, 150 and 300 mmHg. Subjects were asked to keep minute ventilation above FGF. Each of the 18 conditions in each subject (54 conditions in total) was maintained for 3 min, during the last minute of which arterial blood gas was drawn in duplicate and analyzed for $PaCO_2$. Results: The difference (mean ± SD, mmHg) between $tPACO_2$ and $PETCO_2$ was (0.3 +/- 1.3) was not significantly different from zero (p=0.36). $PETCO_2 – PaCO_2$ was -0.5 ± 0.8, which was in the same range as between duplicate $PaCO_2$ measurements (0.4 ± 1.4 mmHg, p=0.96). RR, $tPACO_2$, and $PETO_2$ had no discernable effect on the differences. Conclusion: This is the first report of precisely setting $PACO_2$ over a large range of values as verified by $PaCO_2$ measurements. Under these conditions, $PETCO_2$ was equal to $PACO_2$ ($PaCO_2$) despite a wide range of RR and $PETO_2$.

114. SERUM LACTATE INCREASED WITH MILD EXERCISE IN NATIVE HIMALAYAN HIGHLANDERS. S Ito[1], M Slessarev[1], E Prisman[1], R Watson[2], R Greene[3], T Norboo[4], T Stobdan[4], D Diskit[5], A Norboo[5], M Kunzang[6], J Fisher[1], J Duffin[1], O Appenzeller[7]. *[1]Departments of Anesthesia and Physiology, University Health Network, University of Toronto[2]University of California, San Diego[3]New Mexico Highlands University[4]Ladakh Institute of Prevention and Ladakh Heart Foundation[5]Sonam Norboo memorial hospital Leh, Ladakh[6]Ladakh autonomous hill development council, [7]NMHEMCRF Research Foundation, Albuquerque, NM. Email: sho2ito@yahoo.co.jp*

Introduction: Classically, in exercise at altitude, the limits of aerobic metabolism are exhausted before anaerobic pathways are recruited. This change from aerobic to anaerobic metabo-

lism usually occurs at the anaerobic threshold, just before maximal O_2 consumption (VO_2). We wondered whether Himalayan highlanders have developed the means to recruit anaerobic metabolism at earlier stages of exercise as an adaptation to chronic hypoxia. Methods: We studied 15 male indigenous natives of Ladakh, India aged 30 to 56 living at over 4500 m. The subjects breathed through a sequential gas delivery circuit at rest and while sitting and performing metronome-synchronized knee extensions at a rate of 50/min. Oxygen saturation (SpO_2), end-tidal PCO_2 and end-tidal PO_2 were monitored andVO_2, CO_2 production (VCO_2) and serum lactate concentrations (Lactate) were measured during both conditions. Results: During exercise, VO_2 increased from 6.7 ± 1.9 to 12.8 ± 2.6 ml/min/kg, which is only approximately 30% of the maximal VO_2 expected in an age-matched lowlander at sea level. Lactate increased significantly during exercise (from 0.81± 0.11 to 1.06 ± 0.24 mmol/L, $p<0.01$). The percent increase in lactate was significantly higher in subjects with $SpO_2 > 83\%$ than in those with $SpO_2 < 83\%$ (75.8 ± 56.4 vs 9.8 ± 17.4 %, $p<0.01$). There was no correlation of the extent of Lactate increase and age, hemoglobin and chronic mountain sickness score. Conclusions: In contrast to lowlanders at sea-level, mild exercise in hypoxic conditions increases serum lactate levels in Himalayan highlanders. This effect seems to be more prominent in those with higher resting SpO_2. Earlier recruitment of anaerobic metabolism may be part of the adaptive repertoire to chronic hypoxia in this population.

115. NEW INITIATIVE: GRADUATE PROGRAM IN MOUNTAIN MEDICINE AND HIGH ALTITUDE PHYSIOLOGY. Linda Johannson[1], Esteban Ortiz[1], Taj Jadavji[1], Jon Kolb[3], Buddha Basnyat[4], Fabiola Léon-Velarde[5], Marc Poulin[1,2,3]. *[1]Department of Medical Sciences, Faculty of Medicine, University of Calgary, Calgary AB, Canada, [2]Department of Physiology & Biophysics, Faculty of Medicine, University of Calgary, Calgary AB, Canada, [3]Faculty of Kinesiology, University of Calgary, Calgary AB, Canada, [4]Nepal International Clinic, Kathmandu Nepal, [5]Department of Physiological Sciences, Universidad Peruana Cayetano Heredia, Lima Peru. Email: poulin@ucalgary.ca*

A graduate specialization program in Mountain Medicine and High Altitude Physiology (MMHAP), leading to an MSc in Medical Sciences, is offered at the University of Calgary. The aim is to provide students with a broad foundation in the areas of MMHAP. The specialization program is unique in Canada and the first of its kind in the world that aims to better understand the adaptations associated with the hypoxia of altitude in humans. This program is a result of an exciting initiative that was spearheaded in 2003-2004 by Buddha Basnyat, Taj Jadavji, Jon Kolb, Fabiola Léon-Velarde and Marc Poulin to introduce trainees from mountainous countries (initially, Canada, Nepal and Peru) to study physiological changes and pathophysiology associated with the hypoxia of altitude. Many pathophysiological mechanisms underlying diseases associated with the hypoxia of high altitude (i.e., AMS, CMS, HAPE, HACE) remain elusive and this program will help to shed more light on these problems and others associated with hypoxia and high altitude. The theoretical components will be taught at the University of Calgary and the controlled research studies will be carried out in laboratories in Calgary (i.e., Laboratory of Human Cerebrovascular Physiology, Human Performance Laboratory, Foothills Sleep Laboratory, Seaman Family MR Centre, Experimental Imaging Centre). These components will be complimented by field studies in the mountains of the Nepal Himalayas, the Canadian Rockies and the Peruvian Andes. As the program expands, it is expected that other mountainous countries will become involved and that field studies would take place in those locations as well. We hope this research will be useful for sojourners and high altitude natives. In addition this initiative between the developing and the developed world bonded by mountains will likely benefit both participating trainees and the fields of MMHAP.

116. INCREASED ANTI OXIDANT ACTIVITY DURING PREGNANCY IN NATIVE (ANDEAN) VS. NEWCOMER (EUROPEAN) HIGH-ALTITUDE RESIDENTS. CG Julian[1,2], E Vargas[3], JM McCord[1], S Bose[1], H Yamashiro[4], C Rodriquez[4], A Rodriquez[3], VA Browne[1], MJ Wilson[1,2], LG Moore[1,2]. *[1]Altitude Research Center University of Colorado at Denver and Health Sciences Center, Denver, CO USA[2]Dept of Health/Behavioral Sciences, University of Colorado at Denver and Health Sciences Center, Denver, USA[3]Instituto Boliviano de Biología de Altura, La Paz, BO[4]Clinica Sirani, Sta Cruz, BO. Email: cgjulian@ouray.cudenver.edu*

Oxidative stress, resulting from the greater production of reactive oxygen species relative to antioxidant generation, likely plays an important role in the maternal vascular dysfunction characteristic of preeclampsia and intrauterine growth restriction (IUGR). Objective: To determine whether enhanced antioxidative capacity contributes to the protection from IUGR afforded by multigenerational high-altitude ancestry. Methods: Erythrocyte catalase (CAT) and superoxide dismutase (SOD) activity were measured using spectrophotometric and xanthine oxidase/xanthine/cytochrome c methods respectively in whole blood obtained from 92 women at low (n=46) and high (n=56) altitude of Andean or European ancestry during pregnancy (20w, 36w) and postpartum (3m, pp) for an index of the nonpregnant state. Results: Pregnancy decreased CAT activity at 20w and 36w in both groups at low altitude (Andeans = -23% and -26%, Europeans = -24% and -21%, all $p<0.01$) but did not change SOD. Andeans had higher CAT and SOD activity near term than Europeans at high altitude (CAT=158,221 ± 4562 vs. 112,557 ± 6062 units/ml, SOD=575.8 ±15.7 vs. 484.1 ± 28.3 units/ml respectively, both $p<0.001$), but not at low altitude. Conclusions: Pregnancy lowered erythrocyte CAT relative to pp values, consistent with pregnancy being a state of oxidative stress. Andeans appear better able to increase antioxidant generation compared with European women, perhaps enabling them to achieve 'normal' vascular adaptation to pregnancy and thus protect their offspring from IUGR at altitude. (AHA predoctoral fellowship to CGJ, NSF predoctoral fellowship to MJW, NIH-HL079647). Conclusions: Low birth weight increases susceptibility to left-sided cardiovascular disease. The present data suggest that pulmonary structure and function may also be compromised and contribute to the development of CMS. Further study of the consequences of intrauterine/neonatal hypoxia on the maturation of pulmonary structure, function and susceptibility to CMS is warranted. (NIH-HL079647).

117. THE EFFECT OF THE ACE-I/D POLYMORPHISM ON CLIMBERS ATTEMPTING MT KILIMANJARO (5895M). NS Kalson[1], I Tyrell-Marsh[1], AG Whitehead[1], H Frost[1], S Stokes[1], MD Earl[1], A Gibbs[2], H Montgomery[3], AJ Davies[1]. *Manchester Altitude Research Society [2] University of Manchester [3]University College London. Email: nickkalson@yahoo.co.uk*

We examined whether the ACE-I/D genotype influenced summiting success and Acute Mountain Sickness (AMS) occurrence in a non-elite population of climbers on Mt Kilimanjaro (5895m). A polymorphism of the human angiotensin converting enzyme (ACE) gene has been identified which is characterised by the Insertion (I allele) or Deletion (D allele) of a 287bp fragment. The I allele is associated with lower enzymatic activity and an increased I allele frequency has been noted in elite endurance athletes and elite high altitude mountaineers. 286 climbers attempting to reach the summit of Mount Kilimanjaro were included in the study. Participants ascended from 1860m to the summit (5895m) over five or six days along a fixed ascent profile. Genotype was determined in each climber, and each night the climbers were assessed for AMS using the Lake Louise Scoring (LLS) system. ACE genotype was determined in 286 climbers (65 II, 150 ID, and 71 DD). Allele frequency was in Hardy-Weinberg equilibrium (P=0.628). Genotype did not influence summit success (66% of II, 58% of ID and 67% of DD; P = 0.575); the I allele frequency was 0.46 for those reaching the summit and 0.48 for those not. More than

70% of climbers suffered AMS (LLS >4), but the severity of AMS as defined by the LLS was not associated with ACE genotype on any days of the climb (range P=0.244 for day 1 to P=0.824 for day 6). The I allele frequency in those with severe AMS (LLS > 10) was not different to those without AMS (LLS<4) (0.46 compared with 0.47). We conclude that the ACE-I/D polymorphism has no important effect on developing AMS or on successfully summiting Mt Kilimanjaro.

118. PLASMA ERYTHROPOIETIN CONCENTRATION IN HUMANS IS RELATED MORE TO HYPOXEMIA THAN TO HYPOXIA. Carsten Lundby. *Department of Sport Science, Århus University, Denmark. Email: lundby@idraet.au.dk*

The main stimulus for Epo synthesis is generally thought to be decreasing intracellular PO_2 within the renal cortex. This mechanism however, does not fit with studies conducted in humans at altitude. With acclimatization PaO_2 remains depressed whereas arterial oxygen content (CaO_2) and plasma Epo concentration gradually return toward sea level values. This may indicate that CaO_2 could be the main regulator of Epo production, and this hypothesis was tested with the present study. In a set of single blinded, placebo, cross over experiments, eight human subjects breathe either 1) room air (normoxia) 2) 11% O_2 balanced in nitrogen which lowered CaO_2 and PaO_2 (hypoxia) or 3) Carbon monoxide + normoxia which lowered CaO_2 but kept PaO_2 high (hypoxemia). Arterial blood samples were obtained before, as well as during and after the initiation of five hours of gas mixture breathing. In the two hypoxic conditions CaO_2 was on average decreased to similar levels (16.4 ± 0.1 ($p<0.05$) and 15.9 ± 0.3 ($p<0.05$) ml.dl-1 hypoxia and hypoxemia, respectively), whereas PaO_2 was only reduced ($p<0.05$) with hypoxia. Compared to the normoxic control experiments plasma Epo was increased in both conditions after 120 min of exposure (normoxia: 11.5 ± 4.3, hypoxia: 17.2 ± 7.8 ($p<0.05$), hypoxemia: 17.8 ± 5.5 ($p<0.05$) µl.l-1), and were also augmented to a similar extend after five hours of gas mixture breathing (normoxia: 11.6 ± 4.1, hypoxia: 29.3 ± 8.2 ($p<0.05$), hypoxemia: 25.3 ± 7.8 ($p<0.05$)µl.l-1). This may rule out that changes in PaO_2 are the main regulator of Epo synthesis, and could suggest that CaO_2 is the critical value regulating Epo production.

119. SKELETAL MUSCLE CELLS AND ERYTHROPOIETIN: GROWTH AND DIFFERENTIATION. EFFECT OF HYPOXIA. Thierry Launay, Séverine Divoux, Dominique Marchant, Nicolas Bourdillon, Fabrice Favret, Alain Duvallet, Jean-Paul Richalet, Michèle Beaudry. *Université Paris 13 EA 2363 "Réponses cellulaires et fonctionnelles à l'hypoxie" Bobigny France. Email: mbeaudry@noos.fr*

Erythropoietin (Epo) has been described in litterature for its biological effects in various cells and tissues. Recently, the role of Epo on skeletal muscle cells has been discussed. In murine cultured muscle cells, Epo was shown to enhance myoblast proliferation and reduce differentiation. The aim of this work was first to evaluate the effects of Epo as a growth factor to increase the accumulation of myogenic cells in human primary myoblast cultures in both normal condition cultures and hypoxia conditions cultures. These results beeing of great interest in muscle pathology therapies. Cells were grown in standard medium supplemented or not with Epo, in 21% or 1% oxygen conditions respectively. The Epo receptor (EpoR) expression was determined using RT-PCR and western blotting, cell differentiation by immunostaining using mogenin and fast myosin heavy chain as a marker .This work confirms the expression of both EpoR mRNA and protein in both rat and humuan an skeletal muscle cells. In 21% oxygen conditions, Epo (1 to 10 u/ml) does not modify both proliferation and differentiation. In hypoxia conditions, proliferation is reduced and differentiation inhibited, these results are not modified when muscle cells are grown with Epo (1 to 10 u/ml). Our results demonstrate that on the contrary of results published by others using primary mouse satellite cells and mouse cells from the C2C12 line, Epo does not activate the proliferation of both rat and human muscle cells neither inhibits the differentiation of

these cells. Because of these results, it has been suggested in many publications that Epo could be involved in the muscle development, repair, by increasing the accumulation of myogenic precursor cells. Our work does not support these hypothesis. Supported by AFM (Association Française contre les Myopathies).

120. THE ROLE OF THE ALTITUDE LEVEL ON CEREBRAL AUTOREGULATION IN MAN RESIDENT TO HIGH ALTITUDE. Gerard F. A. Jansen[1], Anne Krins[1], Buddha Basnyat[2], Joseph A Odoom[1], Can Ice[3], Sanju Lama[4]. *[1]Department of Anesthesiology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands, [2]Nepal International Clinic, Kathmandu, Nepal, [3]Department of Physiology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands, [4]Department of Medical Science, University of Calgary, Calgary, Alberta, Canada. Email: slama@ucalgary.ca*

Previous studies have demonstrated that cerebral autoregulation is impaired in Himalayan high-altitude residents who live above 4200 m. This study was undertaken to determine the altitude at which this impairment of autoregulation occurs. A second aim of this study was to test the hypothesis that administration of oxygen can reverse this impairment in autoregulation at high altitudes. In four groups of 10 Himalayan high-altitude dwellers residing at 1330 m, 2650 m, 3440 m and 4243 m, oximetry (SaO_2), blood pressure and middle cerebral artery blood velocity were monitored during infusion of phenylephrine to determine static cerebral autoregulation. Based on these measurements the cerebral autoregulation index (AI) was calculated. Normally, AI is between zero and 1, and AI<0.4 implies absent autoregulation. At 1330 m (SaO_2=97%), 2650 m (SaO_2=96%) and 3440 m (SaO_2=93%), AI was intact with values of 0.63±0.27, 0.57±0.22 and 0.57±0.15 respectively. At 4243 m (SaO_2=88%), AI was 0.22±0.18 (P<0.0005, compared with AI at the lower altitudes), but increased to 0.49±0.23 (P=0.008, paired t test) when oxygen was administered (SaO_2=98%). In conclusion, high-altitude residents living at 4243 m show impaired cerebral autoregulation, which improved during oxygen administration. Those living at 3440 m and lower have intact cerebral autoregulation. This study showed that the altitude region between 3440 m and 4243 m, which is marked by oxygen saturations in the high-altitude dwellers between 93% and 88%, is a transitional zone, above which cerebral autoregulation is impaired and below which autoregulation is intact.

121. SIMILARITY OF IBUPROFEN AND ACETAZOLAMIDE IN THE PREVENTION OF HIGH ALTITUDE HEADACHE: THE HEADACHE EVALUATION AT ALTITUDE TRIAL (HEAT). GS Lipman[1], JH Gertsch[2], PS Holck[3], B Basnyat[4], AL Merritt[5], EA Weiss[6], for the HEAT group7. *[1]Department of Emergency Medicine, Stanford University Hospital, [2]Department of Neurology, Stanford Univeristy Hospital, [3]Department of Public Health Sciences and Epidemiology, University of Hawaii, [4]Himalyan Rescue Association, [5]Keck School of Medicine, University of Southern California, [6]Department of Emergency Medicine, Stanford University Hospital, 7Affiliated Programs. Email: grantlip@hotmail.com*

Context: Several small trials have reported that non steroidal anti-inflammatory medicines (NSAID) may effectively prevent high altitude headache (HAH). To date there have been no large randomized trials comparing NSAIDs with acetazolamide, the most effective prophylaxis for acute mountain sickness (AMS), of which headache is a central defining factor. Objective: To evaluate ibuprofen and acetazolamide's efficacy as prophylaxis against HAH. Design: Double-blind, randomized, placebo-controlled trial conducted from October-November 2005. Settings & Participants: Participants were western trekkers enrolled at Pherich/Dingboche at 4280m/4358m on the Mount Everest base camp approach. The study endpoint was at Lobuje, 4928m. 343 healthy trekkers were enrolled and 265 completed the trial. Interventions: Participants were randomly assigned to receive Ibuprofen 800mg, acetazolamide 85mg, or visually matched placebo

taken three times a day, with at least 3 doses before ascent. Main Outcome Measures: A 100mm Visual Analog Scale (VAS), Lake Louise AMS Score (LLQ), and Environmental Symptoms Questionnaire version III-C (ESQ III-C) were used to evaluate HAH incidence and severity. Secondary outcomes measured AMS incidence, severity, and blood oxygen saturation. Results: The three treatment arms were similar in regard to demographics. Acetazolamide was not significantly different from ibuprofen for preventing headache incidence (34.4% v 34.3%; p= 0.99), and neither treatment was efficacious in prophylaxis over placebo (42.2%; p=0. 26). However, headaches described by LLQ as 'moderate' or 'severe' were prevented to a significant degree by ibuprofen over acetazolamide (3.9% v 11.5% p = 0.05). This result was supported by findings approaching significance by VAS of > 13mm (11.3% v 15.8%; p= 0.08). Conclusions: The study was not able to demonstrate a clear benefit of either acetazolamide or ibuprofen in preventing HAH. However, it suggests that ibuprofen minimizes the incidence of more severe headaches at altitude.

122. ALL THAT GURGLES AT HIGH ALTITUDE IS NOT HAPE. Andrew M. Luks, Erik R. Swenson. *Division of Pulmonary and Critical Care Medicine University of Washington Seattle, Washington. Email: aluks@u.washington.edu*

When an athlete, trekker or climber develops pulmonary edema at high elevations, it is tempting to attribute the problem to the altitude alone and call it a case of High Altitude Pulmonary Edema (HAPE). However, other critical factors may play a role. We present the case of a 38 year-old participant in the Bicycle Race Across America who developed severe pulmonary edema while cycling at an altitude of 2,380 meters on the fourth day of the race. With hospitalization and standard support for what appeared to be a classic case of HAPE, he made a quick and full recovery. A post-race work-up revealed no evidence of underlying cardiopulmonary disease or susceptibility to HAPE. We hypothesize that his excessive daily sodium intake (23 - 25 grams or 1000 – 1100 mEq) over the course of the race likely led to an expanded extracellular volume, increased hydrostatic pressure, and decreased oncotic pressure. These factors, in combination with ambient hypoxia, elevated cardiac output and reduced renal perfusion expected with sustained, high-level exercise, may have led to the development of acute pulmonary edema. This case highlights the pitfalls of overly aggressive sodium intake during endurance races at high altitude and demonstrates the critical role that other factors beyond the ambient hypoxia and innate susceptibility may play in the development of pulmonary edema at high elevations.

123. CEREBRAL HEMODYNAMICS DURING HYPERCAPNIA IN YOUNG AND POSTMENOPAUSAL WOMEN. Georgios D. Mitsis[1, 2], Chantel D. Debert[3, 4], Mahmoud Hajo[5], Vasilis Z. Marmarelis[5], Marc J. Poulin[3,4,6,7]. *[1]Institute of Communication and Computer Systems, National Technical University of Athens, Athens, Greece, [2]fMRIB Centre, University of Oxford, Oxford, United Kingdom, [3]Department of Physiology & Biophysics, University of Calgary, Calgary, Alberta, Canada, [4]Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, Canada, [5]Department of Biomedical Engineering, University of Southern California, Los Angeles, CA, USA, [6]Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada, [7]Faculty of Kinesiology, University of Calgary, Calgary, Alberta, Canada. Email: gmitsis@fmrib.ox.ac.uk*

Introduction: Cerebral blood flow regulation becomes impaired with cardiovascular disease and aging. Here, we examine cerebral hemodynamics (dynamic pressure autoregulation and CO_2 reactivity) in pre- and post-menopausal women during baseline and hypercapnic conditions. Methods: Six premenopausal (PreM) and thirteen postmenopausal women, six of whom were receiving hormonal therapy(PM-HT) and seven who were not (PM-noHT), were exposed to hypercapnia (end-tidal PCO_2 ($PETCO_2$)=+8 Torr above resting values), induced by end-tidal

forcing, for 20 min. Mean arterial blood pressure (MABP) and middle cerebral artery blood flow velocity (MCAV) were measured by photoplethysmography and transcranial Doppler ultrasound respectively. Linear impulse response (IR) and nonlinear Volterra models were employed to quantify the dynamics between MABP-MCAV and CO_2-MCAV. Both the MCAV response to the CO_2 step and shorter data-sets of spontaneous fluctuations around the mean during baseline, hypercapnia and post-hypercapnia were examined. Results: Baseline MABP and MCAV were not significantly different between the three groups and $PETCO_2$ was higher in PM-noHT women. All hemodynamic parameters increased significantly during hypercapnia. MCAV decreased significantly post-hypercapnia, while the decrease in MABP was significant in PM-HT women only. However, both remained significantly higher than baseline, in contrast to $PETCO_2$. Nonlinear models improved prediction errors in all cases. Dynamic CO_2 reactivity to the hypercapnic step, assessed by the $PETCO_2$-MCAV IRs and Volterra kernels, was slightly decreased in PM women. Dynamic pressure autoregulation, i.e., MABP-MCAV IRs and kernels, and CO_2 reactivity to spontaneous fluctuations were similar between groups. Reactivity to spontaneous CO_2 changes and low-frequency MCAV variability decreased during hypercapnia in all groups. Conclusions: Cerebral hemodynamics were not affected significantly in PM women. On the other hand, dynamic reactivity to CO_2 spontaneous fluctuations reduced during hypercapnia in PreM and PM women. This study was funded by the European Social Fund and National Resources-General Secretariat for Research and Development(Program ENTER04), AHFMR, HSFA and CIHR.

124. UPREGULATED ENDOTHELIN RECEPTORS IN CIRCULATING ENDOTHELIAL CELLS IN HAPE? Heimo Mairbäurl[1], Marc Berger[2], Peter Bärtsch[1], Christoph Dehnert[1], Guido Schendler[1], Elmar Menold[1], Andy Luks[3], Damian Bailey[4], Vitalie Faoro[5], Christian Castell[1], Erik Swenson[3]. *[1]Medical Clinic VII, Sports Medicine, University Hospital Heidelberg, Germany[2]Department of Anesthesiology, University Hospital Heidelberg, Germany[3]Medical and Research Services, Veterans Affairs Puget Sound Health Care System, University of Washington, Seattle, USA[4]Department of Physiology, University of Glamorgan, UK[5]Laboratoire de Physiopathologie, Université Libre de Bruxelles, Belgium. heimo.mairbaeurl@med.uni-heidelberg.de*

HAPE is associated with exaggerated pulmonary hypertension which enhances the extravasation of plasma and blood cells into the lung interstitial and alveolar space. The mechanisms for the disproportionate vasoconstriction are not clear. A decreased bioavailability of NO and endothelial dysfunction with an impairment of the NO-system in hypoxia only have been discussed as possible mechanisms. We hypothesized that endothelial dysfunction could be demonstrated in circulating endothelial cells and therefore measured the expression of vasoactive substances in circulating endothelial cells in individuals developing HAPE in the Capanna Margherita (4559m). Before and 22h after ascent to 4559m blood was collected and circulating endothelial cells were isolated with magnetic beads coated with anti-CD146 antibodies. Cells were lyzed and frozen for analysis by RT-PCR. The results indicate that more cells could be extracted at high altitude than at sea level. The lack of increase in GAPD and caspase-9 mRNA indicates that cells were not hypoxic and apoptotic, respectively. VEGF was unchanged, whereas IL-6 mRNA tended to be decreased in all individuals indicating lack of stimulated angiogenesis and inflammation. eNOS (NOS3) mRNA tended to be increased equally in controls and HAPE, whereas the endothelin-1 mRNA tended to be increased in HAPE only. ET-receptor mRNA could not be detected in controls at low and high altitude. However, in HAPE both receptor mRNAs, ETA and ETB, were detectible at high altitude only. These results indicate no impairment of eNOS expression in HAPE but an upregulation of ET-receptors in hypoxia which might indicate an increased sensitivity to this vasoconstricting, endothelium-controlled mediator.

125. SUSCEPTIBILITY TO ARTERIAL O_2 DESATURATION ($DSAO_2$) AND ACUTE MOUNTAIN SICKNESS (AMS) DURING ASCENT OF KILIMANJARO. Pierre Mayer, Antoine Delage, Claude Poirier, Vincent Jobin, François Bellemare. *Centre Hospitalier de l'Université de Montréal, Canada. Email: pierre.mayer@umontreal.ca*

AMS affects 25-85% of individuals going to altitudes >2500m. A low ventilatory response to hypoxia was suggested as a major predisposing factor but conflicting reports emerged as to whether dSa O_2 at altitude can be predicted from sea-level hypoxic challenges. METHODS: To assess $dSaO_2$ susceptibility at altitude, poikilocapnic hypoxic challenges (11% O_2, balance N2) were administered before the ascent to 10 normal volunteers (1) for 20 minutes while resting supine (R challenge) and (2) during incrementing exercise to 50% VO_2Max (E challenge). During ascent, SaO_2 was recorded continuously while climbing and asleep by reflectance forehead pulse oximetry (PalmSat 2500, 8000R sensor, Nonin Medical Inc, MN, USA). AMS was assessed daily (Lake Louise Questionnaire). RESULTS: Mean SaO_2 during climbing and sleep for the group never differed significantly from each other. Both values for the group declined linearly with increasing altitude >3000m and correlated significantly with average AMS score (r2 = 0.85). On the last day of ascent, mean SaO_2 during climbing episodes (Altitude: 5.3km) and during sleep (Altitude: 5.6km) were 69.7±5.9% and 68.7±5.2%, respectively. When compared to these levels of $dSaO_2$, the predictive value of R challenge SaO_2 was 102.5±9.9% (range 89-117%) during sleep and 101.8±12.1% during climbing (range 80-116%). The predictive value of E challenge SaO_2 was 93.7±8.5% (range 74-106%) during climbing and 93.0±13.7% (range 78-113%) during sleep. CONCLUSION: A close relationship was found for the group between AMS score and the severity of hypoxemia at high altitude whether recorded while climbing or asleep. Sea-level poikilocapnic hypoxic challenges are useful when assessing individual susceptibility to $dSaO_2$ at comparable levels of hypobaric hypoxia. Supported by Laboratoire Médical Biron.

126. CHANGES IN CEREBRAL KU70 EXPRESSION FOLLOWING CEREBRAL HYPOXIA/ISCHEMIA IN NEONATAL RATS. S. Meng[1], M. Qiao[1], S. Crowley[2], N. Webster[2], S. Lama[2], U.I. Tuor[1,2]. *[1]Institute for Biodiagnostics (West), NRC, Calgary., [2]Faculty of Medicine, University of Calgary, Alberta, Calgary. Email: slama@ucalgary.ca*

Ku 70, a subunit of the heterodimeric protein Ku, has been shown to have a role in triggering a DNA repair pathway following oxidative stress. There is also some evidence for changes in its expression after cerebral ischemia and reperfusion in adult rats. However, the potential role for Ku70 in recovery of neonatal brain from a hypoxic-ischemic insult is not known. We hypothesized that there would be changes in Ku70 protein expression following neonatal cerebral hypoxia-ischemia consistent with its role in DNA repair. Thus, changes in the expression of Ku70 were investigated in 17 neonatal (7 day old) rats at various times following a hypoxic-ischemic insult (surgical ligation of the right carotid artery and then exposure to 70 minutes of hypoxia - 8% oxygen/92% nitrogen). Animals were euthanized 1 day, 3 days, 1 week, 4 weeks and 9 weeks post insult (n=3-4/group). Changes in Ku70 protein were investigated using immunohistochemistry and cellular localization was determined with double labeling with NeuN (neurons), GFAP (astrocytes), DAPI (nucleus) or BrdU (cell proliferation). Ku70 protein expression was increased in both the core infarct and the peri-infarct region 24hrs post hypoxia/ischemia. After 72 hrs and by 1 week, increased Ku70 expression was predominantly in the peri-infarct region. Staining was predominantly nuclear with some co-localization in neurons and astrocytes. At 9 weeks post hypoxia-ischemia there was frequent co-localization of Ku70 expression with cells positive for BrdU in the perinfarct region. The early increased expression and persistence of Ku70 positive cells at 9 weeks after hypoxia/ischemia, and its localization in newly formed cells, suggest a potential role for Ku70 protein in repair and recovery after hypoxic/ischemic injury to brain in neonates. This study was funded by the Robertson fund for Cerebral Palsy, Heat And

Stroke Foundation of Alberta and the AHFMR.

127. THE CARDIAC OUTPUT RESPONSE TO NEGATIVE INSPIRATORY PRESSURE DURING ORTHOSTATIC STRESS. Alexandra Mardimae[1,2], Cathie Kessler[2], Shoji Ito[2], Jay Han[1,2], Marat Slessarev[1,2], Jim Duffin[1], Lorne Chi[3,4], Milos Popovic[3,4], Joseph Fisher[1,2]. *[1]Department of Physiology, University of Toronto, [2]Department of Anaesthesia, University Health Network, [3]Institute of Biomaterials and Biomedical Engineering, University of Toronto, [4]Toronto Rehabilitation Institute, University of Toronto. Email: a.mardimae@utoronto.ca*

Introduction: It has been argued that inspiratory impedance, resulting in negative inspiratory pressure (NIP), may improve venous return and thereby maintain cardiac output and blood pressure during orthostatic stress. However, this has not been well studied. The purpose of this study is to evaluate the cardiac output response to a head-up tilt with and without NIP. Methods: In a prospective study, ten healthy males underwent two 70º head-up tilts while breathing through a sequential rebreathing circuit with inspiratory impedance of 0 (Sham) and -9 cm H2O (NIP), in random order. Cardiac output was measured using a non-invasive CO_2-based differential Fick method. Heart rate and blood pressure were measured continuously throughout the testing protocol. Results: On head-up tilt, cardiac output fell on average by 32.2 ± 9.2 % with Sham and 28.0 ± 12.9 % with NIP ($p = 0.5$). Average mean blood pressure increased by 4.5 ± 8.2 % with Sham, and 6.5 ± 7.1 % with NIP ($p = 0.6$). Heart rate also increased equally with both circuits (average 37.5 ± 7.1 % with Sham, to 35.9 ± 9.4 % with NIP, $p = 0.7$). Conclusions: The hemodynamic effects of a head-up tilt in healthy subjects were similar when breathing through the Sham and NIP devices. However, NIP could still be beneficial as a rescue maneuver in orthostatically challenged healthy subjects just prior to fainting, or in patients prone to orthostatic hypotension (i.e. spinal cord injury).

128. HIGH ALTITUDE SEIZURES: THE EPIDEMIOLOGY OF AN ACUTE SYMPTOMATIC SEIZURE. Edward Maa[1], Robert Roach[2], Michael Patz[1], Benjamin Honigman[2], Mark Spitz[1]. *[1]Division of Epilepsy, Department of Neurology, University of Colorado Health Sciences Center, [2]Altitude Research Center, University of Colorado Health Sciences Center.Email:edward.maa@uchsc.edu*

RATIONALE: Twenty million people vacation in the American Rockies annually. The physiologic consequences of transient environmental hypoxia have been responsible for everything from poor sleep to pulmonary edema. Anecdotally, first-time seizures have erupted in this setting. Epidemiologic data for high altitude seizures at the moderate altitudes (> 3300 m) of our American resort towns does not exist. METHODS: A retrospective chart review examining the risk factors in subjects with new or worsening seizures upon arrival to the moderate altitudes of Colorado resort towns was performed. Electronic records from Summit County Emergency Department (a cachement area including Breckenridge, Arapahoe Basin, and Keystone ski resorts) were reviewed from October 2001 to October 2005 for the ICD9 diagnosis codes for seizures and epilepsy. RESULTS: A total of 64 individual subjects suffered one or more tonic-clonic seizures, 28 occurring in subjects visiting from elevations less than 3300 m, designated High Altitude Seizures (HAS). Subjects with HAS had an average POx reading of 82.6%. Subjects with Local Seizures (LS) averaged POx of 87.3%. Alcohol abuse and intracranial pathology discovered by imaging was significantly underrepresented in HAS subjects. Headaches, sleep disturbances, and hypoxemia at presentation were more frequent in the same population. Summit County's 6M annual visitors translates to an incidence of HAS 0.09 per 100,000 visitors. CONCLUSION: Subjects who experienced new or worsening seizures, from elevations less than 8000 ft, were more likely to show signs of hypoxia-related physiologic changes. Compared with subjects from el-

evations of 3300 m or greater, these subjects demonstrated poorer oxygen saturations, increased tachycardia, and more disordered sleep and headaches. Transient exposure to hypobaric hypoxia is suspected of altering cerebral physiology in such a way as to increase the risk of developing a seizure. Mechanisms are yet unknown, and further research, including a prospective collection of epidemiologic data is required.

129. INCREASED METABOLIC ACTIVITY OF LEUKOCYTES IN AMS-SUSCEPTIBLES. Heimo Mairbäurl[4], Vitalie Faoro[1], Damian Bailey[2], Marc Berger[3], Peter Bärtsch[4], Christoph Dehnert[4], Guido Schendler[4], Elmar Menold[4], Andy Luks[5], Christian Castell[4], Erik Swenson[5]. *[1]Laboratoire de Physiopathologie, Université Libre de Bruxelles, Belgium[2]Department of Physiology, University of Glamorgan, UK[3]Department of Anesthesiology, University Hospital Heidelberg, Germany[4]Medical Clinic VII, Sports Medicine, University Hospital Heidelberg, Germany[5]Medical and Research Services, Veterans Affairs Puget Sound Health Care System, University of Washington, Seattle, USA*

Hypoxia has been shown to inhibit cellular metabolism in different cell types in vitro which has been associated with an impairment of cell function. We studied whether this also occurs during in vivo hypoxia at high altitude and whether there is a difference between individuals with AMS in the Capanna Margherita (4559m; HA). In a subset of subjects blood was collected before (LA) and ~22h after ascent to HA and mononuclear leukocytes (MNL) were isolated by density gradient centrifugation. At either location, cellular respiration was measured at a PO_2 of 100 (normoxia) and 20mmHg (hypoxia) in a micro-respirometer in intact cells and after permeabilization to assess whole cell and mitochondrial metabolism, respectively. The results indicated that acute hypoxia at LA inhibited cellular respiration (-40%) and mitochondrial activity (-40 to -70%) in MNL of all subjects. Acute reoxygenation restored activity. Stimulation with PMA significantly increased respiration but inhibition by hypoxia persisted. At HA respiration was decreased significantly even during normoxia, whereas acute hypoxia had no further effect. At LA oxygen consumption of MNL at 100 and 20 mmHg PO_2 was 2 to 3-times higher in subjects who later developed AMS at HA compared to controls whereas at HA values were not different. This effect was also seen in the activity of mitochondrial complexes I, II and III. The generation of ROS of MNL measured by CMH-ESR was higher in AMS at LA than in controls. Acute and HA-hypoxia did not affect ROS production. These results show that both acute and HA exposure to hypoxia cause a decrease in the metabolic activity of MNL. They also show a higher cellular metabolism before ascent to high altitude in subjects who later developed AMS than controls, which has its origin in a higher mitochondrial metabolic activity. Mechanisms that cause this difference are not clear.

130. INDIRECT MARKERS OF PULMONARY ENDOTHELIAL FUNCTION CORRELATE WITH PULMONARY ARTERY PRESSURE AT HIGH-ALTITUDE. Marc Moritz Berger[1,2], Peter Bärtsch[2], Andrew Luks[3], Damian Bailey[4], Christian Castell[2], Guido Schendler[2], Elmar Menold[2], Vitaly Faoro[5], Heimo Mairbäurl[2], Erik Swenson[3], Christoph Dehnert[2]. *[1]Department of Anesthesiology, University of Heidelberg, Germany[2]Department of Internal Medicine VII, Division of Sports Medicine, University of Heidelberg, Germany[3]Department of Medicine, University of Washington, Seattle, USA[4]Department of Physiology, University of Glamorgan, UK[5]Department of Pathophysiology, University of Brussels, Belgium. Email: marc_berger@med.uni-heidelberg.de*

High-altitude induced pulmonary hypertension plays a major role in the development of high-altitude pulmonary edema (HAPE). We hypothesized that the rise in pulmonary artery pressure (PAP) at high-altitude is related to impaired pulmonary vascular endothelial function with reduced nitric oxide (NO) and enhanced endothelin-1 (ET-1) bioavailability. 34 mountaineers were

investigated at low-altitude (110m) and after rapid ascent to high-altitude (<24 hours to 4559m). 3-4 hours after arrival at high-altitude blood samples were drawn via a central venuous and a radial artery catheter, respectively, for determination of plasma nitrite (measured by flow injection analysis technique), plasma ET-1 (measured by radioimmunoassay), and plasma catecholamines (noradrenaline, adrenaline measured by HPLC). Systolic PAP (PASP) was estimated by transthoracic doppler-echocardiography, and chest radiography was used to diagnose pulmonary edema. In subjects developing HAPE the presented data were collected before its onset. Data are given as Mean±SEM. After ascent to high-altitude PASP increased from 23±0.7 mmHg to 37±2 mmHg ($p<0.001$; range at high-altitude: 28-65 mmHg). HAPE developed in 4 participants. Central venuous ET-1 plasma levels increased about 3-fold ($p<0.001$) while plasma nitrite remained stable ($p=0.33$). At low-altitude arterial-central venous (ACV) plasma gradients were negative for ET-1 and positive for nitrite ($p<0.001$). They reversed after ascent to high-altitude ($p<0.05$) and correlated with PASP (ET-1: $R=0.49$, $p<0.001$; nitrite: $R=-0.21$, $p<0.05$). ACV-differences of plasma ET-1 and plasma nitrite showed an inverse correlation ($R=-0.48$; $p<0.001$). Central venuous plasma levels of noradrenaline and adrenaline increased about 2-fold ($p=0.001$) and 1.6-fold ($p<0.05$), respectively, but showed no correlation with PASP. The inversion of the ET-1 and nitrite ACV plasma gradients at high-altitude is compatible with an impaired pulmonary endothelial function with increased pulmonary ET-1 and decreased pulmonary NO bioavailability. The inverse correlation between the ACV gradients of ET-1 and nitrite is in line with a reciprocal pulmonary regulation of ET-1 and NO.

131. CEREBROVASCULAR RESPONSES TO EXOGENOUS NO AT ALTITUDE AND DURING INDUCED HYPEROXIA IN LADAKH HIGHLANDERS. E Prisman[1], M Slessarev[1], S Ito[1], R Watson[2], D Preiss[1], A Vesely[1], D Greene[3], T Norboo[4], T Stobdan[4], D Diskit[5], A Norboo[5], M Kunang[6], JA Fisher[1], O Appenzeller[7]. *[1]Departments of Anesthesia and Physiology, University Health Network, University of Toronto[2]University of California, San Diego[3]New Mexico Highlands University[4]Ladakh Institute of Prevention and Ladakh Heart Foundation[5]Sonam Norboo memorial hospital Leh, Ladakh[6]Ladakh autonomous hill development council, [7]NMHEMCRF, Albuquerque, NM. Email: eitan.prisman@utoronto.ca*

BACKGROUND: Ambient hypoxia induces NO-mediated vasodilatation. We studied the cerebrovascular responses to exogenous NO in Ladakh highlanders while breathing ambient air at 4550m and during induced hyperoxia at the same altitude. METHODS: Ten altitude-native Ladakhi men were examined near their native village. We assessed CMS scores, middle cerebral artery (MCA) velocity (MCAV), internal carotid artery (ICA) velocity (ICAV), ICA diameter (ICAD), oxygen saturation (SaO_2) and blood pressure (BP). MCA resistance (rMCA) was calculated as mBP/MCAV. Measurements were recorded at rest, 7 minutes after administration of exogenous-NO (5 mg nitroglycerine sublingually), 10 minutes of breathing a hyperoxic gas mixture, and 7 minutes after administration of the same dose while hyperoxic. RESULTS: There was a significant decrease in MCAV ($p<0.001$), ICAV ($p<0.001$) and mean arterial BP (mBP) ($p<0.01$), when comparing baseline to exogenous-NO. However, during hyperoxia, there was no significant change in MCAV, ICAV or mBP with administration of exogenous-NO. When comparing baseline with induced hyperoxia, in the absence of exogenous-NO, there was a significant decrease in MCAV ($p<0.001$) and ICAV ($p<0.001$). ICAD did not significantly change at any time. A trend toward an increased rMCA with exogenous-NO in both ambient air and induced hyperoxia was not statistically significant. CONCLUSIONS: The decrease in MCAV and ICAV with no change in ICAD implies a decrease in cerebral blood flow (CBF) during exogenous NO administration at altitude. We suggest that the responsiveness to exogenous NO in the cerebral circulation is heavily modulated by that of the systemic vasculature. Hyperoxia at altitude, in this population, appears to be a potent vasoconstrictor. In the presence of hyperoxia, ICAV and

MCAV did not significantly decrease with exogenous NO. This suggests that CBF, in this population, was not affected by exogenous NO during induced hyperoxia.

132. NEW NON-INVASIVE FICK CARDIAC OUTPUT ESTIMATION AT 3300 M.
David Preiss, Marat Slessarev, Alexander Ivanoff, Ron Somogyi, Vladimir Slessarev, Joseph Fisher. *Department of Anesthesia, University Health Network. Email: david.preiss@utoronto.ca*

Introduction: Cardiac output (Q) can be calculated from steady state minute CO_2 elimination (VCO_2) and end-tidal PCO_2 ($PETCO_2$) by transiently changing VCO_2 to VCO_2' and observing new pseudo-steady state $PETCO_2$' as follows: $Q = (VCO_2\text{-}VCO_2')/k(PETCO_2'\text{-}PETCO_2)$, where k is the slope of the CO_2 dissociation curve. VCO_2 is classically changed through rebreathing. However, this is unsuitable for spontaneously breathing subjects, especially at altitude where rebreathing during exercise may cause particular respiratory distress. We hypothesized that VCO_2 could be changed through transient voluntary hyperventilation to calculate Q. Methods: Ten healthy, male subjects at 3300 m at Mangilki, Kazakhstan exercised at 25% 50% and 75% of estimated VO_2max on a bicycle ergometer. At each work level, VO_2, VCO_2, $PETCO_2$ measurements were made at spontaneous ventilation and after 20 s of voluntary hyperventilation, from which VCO_2', $PETCO_2$' and subsequent Q were calculated. Results: Five measurements from 3 subjects were inadequate for analysis. A total of 23 tests from 9 subjects were analyzed. Regression analysis yielded $CO = 3.99*VO_2 + 6.9$ ($R=0.94$) and Bland-Altman analysis showed a mean difference of -1.21 ± 1.82 L/min from literature values. Conclusion: The method is simple and can be performed with a regular metabolic cart in the course of measuring VO_2. The method is well tolerated and merits further investigation as a suitable approach for estimating Q in field studies.

133. CEREBROVASCULAR RESPONSES TO EXOGENOUS NO AT ALTITUDE AND DURING INDUCED HYPEROXIA IN LADAKH HIGHLANDERS.
E Prisman[1], M Slessarev[1], S Ito[1], R Watson[2], D Preiss[1], A Vesely[1], D Greene[3], T Norboo[4], T Stobdan[4], D Diskit[5], A Norboo[5], M Kunang[6], JA Fisher[1], O Appenzeller[7]. *[1]Departments of Anesthesia and Physiology, University Health Network, University of Toronto, [2]University of California, San Diego, [3]New Mexico Highlands University, [4]Ladakh Institute of Prevention and Ladakh Heart Foundation, [5]Sonam Norboo memorial hospital Leh, Ladakh, [6]Ladakh autonomous hill development council, [7]NMHEMCRF, Albuquerque, NM. Email: eitan.prisman@utoronto.ca*

BACKGROUND: Ambient hypoxia induces NO-mediated vasodilatation. We studied the cerebrovascular responses to exogenous NO in Ladakh highlanders while breathing ambient air at 4550m and during induced hyperoxia at the same altitude. METHODS: Ten altitude-native Ladakhi men were examined near their native village. We assessed CMS scores, middle cerebral artery (MCA) velocity (MCAV), internal carotid artery (ICA) velocity (ICAV), ICA diameter (ICAD), oxygen saturation (SaO_2) and blood pressure (BP). MCA resistance (rMCA) was calculated as mBP/MCAV. Measurements were recorded at rest, 7 minutes after administration of exogenous-NO (5 mg nitroglycerine sublingually), 10 minutes of breathing a hyperoxic gas mixture, and 7 minutes after administration of the same dose while hyperoxic. RESULTS: There was a significant decrease in MCAV ($p<0.001$), ICAV ($p<0.001$) and mean arterial BP (mBP) ($p<0.01$), when comparing baseline to exogenous-NO. However, during hyperoxia, there was no significant change in MCAV, ICAV or mBP with administration of exogenous-NO. When comparing baseline with induced hyperoxia, in the absence of exogenous-NO, there was a significant decrease in MCAV ($p<0.001$) and ICAV ($p<0.001$). ICAD did not significantly change at any time. A trend toward an increased rMCA with exogenous-NO in both ambient air and induced hyperoxia was not statistically significant. CONCLUSIONS: The decrease in MCAV and ICAV with no change in ICAD implies a decrease in cerebral blood flow (CBF) during exogenous NO

administration at altitude. We suggest that the responsiveness to exogenous NO in the cerebral circulation is heavily modulated by that of the systemic vasculature. Hyperoxia at altitude, in this population, appears to be a potent vasoconstrictor. In the presence of hyperoxia, ICAV and MCAV did not significantly decrease with exogenous NO. This suggests that CBF, in this population, was not affected by exogenous NO during induced hyperoxia.

134. SYMPATHETIC RESTRAINT OF MUSCLE BLOOD FLOW DURING HYPOXIC EXERCISE. Michael Stickland, Curtis Smith, Juan Robles, Benjamin Soriano, Jerome Dempsey, *John Rankin Laboratory of Pulmonary Medicine, University of Wisconsin, Madison. Email: michael.stickland@ualberta.ca.*

We have recently shown that the carotid chemoreceptors contribute to the sympathetic restraint of exercising muscle blood flow. Hypoxia is a potent stimulus of the carotid chemoreceptors, and the sympathetic response to exercise is exaggerated in hypoxia. Accordingly, we investigated whether sympathetic restraint of exercising muscle blood flow was greater in hypoxia as compared to normoxia. Six chronically instrumented (ascending aortic and hindlimb flow probes, terminal aortic catheter) dogs performed mild (2.5mph, 5% grade) and moderate (4.0mph, 10% grade) intensity exercise while breathing room air or hypoxia (PaO_2 ~ 45 mmHg) in the intact control condition, and following systemic alpha-adrenergic blockade (phentolamine). Hypoxia typically caused an increase in cardiac output (CO), hindlimb flow (FlowL) and blood pressure (BP), while total (CondT) and hindlimb conductance (CondL) were only greater in hypoxia at the moderate exercise workload. During both mild and moderate exercise, alpha blockade in normoxia resulted in significant vasodilation as evidenced by increases in CO (10%), FlowL (17%), CondT (33%), CondL (43%) and a drop in BP (18%), with the increase in CondL greater than the increase in CondT. Compared to the normoxic response, blockade in hypoxia resulted in significantly greater increase in CondT (59%), CondL (74%), and a correspondingly greater decrease in BP (34%) from baseline. Despite the local vasodilatory properties of hypoxia, there is considerable hypoxia-induced sympathetic restraint of muscle blood flow during both mild and moderate exercise, which helps to maintain arterial blood pressure. Funding: AHA 0655654Z, NHLBI RO1-HL015469, NSERC.

135. HYPOXIC VENTILATORY RESPONSE: METHODOLOGICAL CONSIDERATIONS RELATING TO ARTERIAL OXYGEN SATURATION. Craig Steinback[1], Marc Poulin[1,2,3,4]. *[1]Physiology & Biophysics[2]Clinical Neurosciences[3]Faculty of Medicine[4]Faculty of Kinesiology, University of Calgary, Calgary Alberta, Canada. Email: cdsteinb@uwo.ca*

The ventilatory response to hypoxia (HVR) is most conveniently described as the linear relationship between ventilation and arterial oxygen saturation (SaO_2), most easily approximated using pulse oximetry (SpO_2). However, conditions affecting the oxygen dissociation curve, such as pH or temperature, may result in erroneous calculations of the HVR using this method. HVR was measured during isocapnic (IH, $PETCO_2$ = + 1 Torr above resting) and poikilocapnic (PH, $PETCO_2$ uncontrolled) hypoxia ($PETO_2$ = 45 Torr). Pulse oximetry was used to assess SpO_2. A secondary calculation of oxygenation (ScO_2) was performed using a known transform: ScO_2 = (((($PO_2$3 + 150·PO_2)-1·23,000) + 1)-1. A significant discrepancy between SpO_2 and ScO_2 at the time of peak ventilation during both IH (85.4 ± 5.6% vs. 81.3 ± 0.9%, $P < 0.01$) and PH (87.1 ± 5.1% vs. 80.5 ± 2.1%, $P < 0.001$), and a subsequent progressive decline in SpO_2 during PH were observed. Over-estimation of SpO_2 early in hypoxia may be attributed to a temporal delay in finger blood flow. The decline in SpO_2 during PH is counter to the curve shift predicted by the Bohr effect and the specific effects of PCO_2 in the lung (dilation) which would facilitate increased SaO_2. These data may be explained by alkalotic vasoconstriction of the finger vasculature, in turn

decreasing measured SpO_2. Based on these findings, we suggest that to ensure accuracy of SpO_2 measures, the hand should be warmed to avoid vasoconstriction. Further, we propose that the linearization of the ventilatory response to hypoxia be performed using ScO_2 via mathematical transform as it represents more accurately the true chemoreceptor stimulus (i.e. PO_2) and is less affected by other physiological artefacts. This study was approved by the local ethics board and supported by AHFMR, HSFA and CIHR.

136. THE CEREBRAL BLOOD FLOW RESPONSE TO CO_2 IN HIMALAYAN HIGHLANDERS WITH AND WITHOUT CHRONIC MOUNTAIN SICKNESS. M Slessarev[1], E Prisman[1], S Ito[1], R Watson[2], D Preiss[1], R Greene[3], T Norboo[4], T Stobdan[4], D Diskit[5], A Norboo[5], M Kunzang[6], JA Fisher[1], J Duffin[1], O Appenzeller[7]. *[1]Departments of Anesthesia and Physiology, University Health Network, University of Toronto[2]University of California, San Diego[3]New Mexico Highlands University[4]Ladakh Institute of Prevention and Ladakh Heart Foundation[5]Sonam Norboo Memorial Hospital Leh, Ladakh[6]Ladakh Autonomous Hill Development Council [7]NMHEMCRF, Albuquerque, NM. Email: marat.slessarev@utoronto.ca*

Introduction: Although the cerebral blood flow (CBF) response to CO_2 has been characterized in sea-level residents as linear, two-linear or exponential, it has not been characterized in highlanders. Furthermore; it has been noted that Andean patients with chronic mountain sickness (CMS) have a decreased CBF response compared to normal healthy highlanders in the hypercapnic range, but not in the hypocapnic range. Methods: We investigated the relationship between CBF and CO_2 in 14 male Himalayan residents of Ladakh, India (altitude 4550m) with (n=6) and without (n=8) CMS using transcrannial Doppler and isoxic modified rebreathing tests at hypoxic (50 mmHg) and hyperoxic (150 mmHg) PO_2 tensions. The CBF response was measured throughout hypocapnic and hypercapnic ranges (PCO_2: 20-50 mmHg). Results: Linear relationships between CBF and CO_2 were observed in both normal and CMS highlanders, and the sensitivities (slopes) determined. There was no difference in the sensitivities of the CBF response (% change from resting) to CO_2 (mmHg change) between normal highlanders (mean ± SEM; hypoxic: 4.9 ± 1.0 %/mmHg; hyperoxic: 3.7 ± 0.4 %/mmHg) and highlanders with CMS (hypoxic: 4.7 ± 0.7 %/mmHg; hyperoxic: 4.2 ± 0.2 %/mmHg; $p = 0.835$). In addition, we observed that the sensitivities of the CBF responses to CO_2 were unaffected by PO_2 tensions in both groups ($p=0.262$). Conclusion: The relationship between CBF and CO_2 in Himalayan highlanders is linear over the hypocapnic-hypercapnic range and appears to be slightly greater than that reported previously in sea-level populations (range 1.8 to 3.4 %/mmHg). This relationship is unaffected by the presence of CMS or different tensions of PO_2; the latter suggests a relative CBF insensitivity to hypoxia in Himalayan highlanders.

137. ALTERED METABOLIC STATE DEFINES ADAPTATION TO HIGH ALTITUDE IN ETHIOPIAN HIGHLANDERS. Marat Slessarev[1], Victoria Claydon[2], Giosué Gulli[3], James Duffin[1], Otto Appenzeller[4], Guta Zenebe[5], Amha Gebremedhin[6], Roger Hainsworth[3], Joseph Fisher[1]. *[1]Departments of Anesthesia and Physiology, University Health Network, University of Toronto,[2]International Collaboration On Repair Discoveries, University of British Columbia,[3]Institute for Cardiovascular Research, University of Leeds, UK Department of Neurology,[4]New Mexico Health Enhancement and Marathon Clinics Research Foundation, USA,[5]Department of Neurology, Yehuleshet Higher Clinic, and,[6]Department of Medicine, University of Addis Ababa, Ethiopia. Email: marat.slessarev@utoronto.ca*

Introduction: Ethiopian highlanders (E) are reported to have haemoglobin concentrations and arterial oxygen saturations (SaO_2) within the ranges observed in sea level dwellers. This attribute is distinctly different from that of Andean (A) and Tibetan (T) highlanders who have higher

[Hb] and lower SaO_2 than sea-level dwellers. We investigated the ventilatory and metabolic parameters of E and compared the results to previously reported data from A and T. Methods: In 9 native male E (age: 35.6±2.7 yrs) we measured end-tidal PCO_2 ($PetCO_2$), end-tidal PO_2 ($PetO_2$), SaO_2, and the ventilatory responses to acute and long-term changes in inspired PO_2 at their native altitude (3660 m) and again 1 day after arrival at 760 m. We also measured the metabolic CO_2 production and O_2 consumption and calculated the respiratory quotient (RQ). Results: The $PetCO_2$ of E (median 38.0 [range: 33.5 to 40.5] mmHg) appeared to be higher than that of A (31.0 [25.0 to34.7] mmHg) or T (30.2 [25.2 to 33.0] mmHg). In contrast to previous reports, we found that the $PetO_2$ and SaO_2 of E was similar to that reported for A and T. The RQ in E (38.0 [33.5 to 40.5] mmHg) appeared to be higher than that of A (31.0 [25.0 to34.7] mmHg) or T (30.2 [25.2 to 33.0] mmHg). E also lacked acute ventilatory responses to changes in PO_2 as indicated by the unchanged $PetCO_2$ during acute changes in inspired PO_2 at both altitudes. However, their resting $PetCO_2$ increased after one night at low altitude indicating the presence of a long-term ventilatory sensitivity to O_2. Conclusions: Ethiopian highlanders have a similar resting $PetO_2$, higher resting $PetCO_2$, and higher RQ compared to other highlanders, which suggests that adaptation of Ethiopians to high altitude may involve a change in their metabolic state.

138. EFFECTS OF ACUTE HYPOXIA ON CEREBRAL AND MUSCLE OXYGENATION DURING INCREMENTAL EXERCISE. Andrew Subudhi, Andrew Dimmen, Robert Roach. *Altitude Reserach Center, University of Colorado Denver Health Science Center and Colorado Springs Campuses. Email: asubudhi@uccs.edu*

To determine if fatigue at maximal aerobic power output was associated with a critical decrease in cerebral oxygenation, thirteen male cyclists performed incremental exercise tests (25 W·min-1 ramp) under normoxic (NORM: 21% FIO_2) and acute hypoxic (HYPOX: 12% FIO_2) conditions. Near infrared spectroscopy (NIRS) was used to monitor concentration (μM) changes of oxy- and deoxy-hemoglobin ($\Delta[O_2Hb]$, Δ[HHb]) in the left vastus lateralis and frontal cerebral cortex (Oxymon, Artinis, The Netherlands). Total Hb was calculated ($\Delta[THb] = \Delta[O_2Hb] + \Delta$[HHb]) and used as an index of change in regional blood volume. Repeated measures ANOVA analyses were performed across treatments and workrates (α=0.05). During NORM, cerebral oxygenation rose between 25 and 75% peak power output (Powerpeak) (inc. Δ $[O_2Hb]$, inc. Δ[HHb], inc. Δ[THb]), but fell from 75 to 100% Powerpeak (dec. $\Delta[O_2Hb]$, inc. Δ[HHb], nc. Δ[THb]). In contrast, during HYPOX, cerebral oxygenation dropped progressively across all workrates (dec. $\Delta[O_2Hb]$, inc. Δ[HHb]), while Δ[THb] again rose up to 75% Powerpeak and remained constant thereafter. Changes in cerebral oxygenation during HYPOX were larger than NORM. In muscle, oxygenation decreased progressively throughout exercise in both NORM and HYPOX (dec. $\Delta[O_2Hb]$, inc. Δ [HHb], inc. Δ[THb]), although $\Delta[O_2Hb]$ was unchanged between 75 and 100% Powerpeak. Changes in muscle oxygenation were also greater in HYPOX compared to NORM. These results demonstrate a large tolerance for change in cerebral oxygenation during exercise, thus do not support the notion that a critical decrease in frontal cortex oxygenation limits exercise in normoxia.

139. VENTILATION, HEMODYNAMICS, AND EXERCISE PERFORMANCE OF ELDERLY MEN AFTER INTERMITTENT HYPOXIA TRAINING (IHT). Tatiana Serebrovskaya[1], Oleg Korkushko[2], Valeriy Shatilo[2], Vadim Ischuk[2], Fred Downey[3]. *[1]Bogomoletz Institute of Physiology, Ukraine, [2]Institute of Gerontology, Ukraine, [3]University North Texas Health Science Center, USA. Email: sereb@mail.kar.net*

The efficacy and safety of IHT was investigated in healthy, 60-74 yr men. Fourteen men (Group I) exercised daily 20-30 min (peak submaximal O_2, 23.7±1.0 ml/min/kg); 21 men (Group II) avoided exercise (peak O_2, 18.2±1.3 ml/min/kg, $P < 0.05$). Ventilation, arterial pressure (BP),

heart rate (HR), SaO_2, and ECG were recorded. Before and after 10 days of IHT, the ventilatory response to sustained hypoxia (SH, 12% O_2 for 10 min), work capacity (bicycle ergometer) and forearm cutaneous perfusion (laser Doppler) were determined. IHT (9 min hypoxia, 5 min normoxia, 4/day, isocapnic, hypoxic rebreathing technique) reduced SaO_2 to 81.0±0.6%. Initial SH caused BP to increase >30% in 2.5 % of subjects; these subjects were excluded. During SH no negative ECG changes were observed, and ventilatory response was unaltered by IHT. In Group I, IHT produced no changes in hemodynamic indices and work capacity. In Group II, IHT decreased BP by 7.9±3.1 mm Hg ($P<0.05$), increased submaximal work by 11.3% ($P<0.05$), and increased anaerobic threshold O_2 by 12.7 % ($P<0.05$). The increase in HR and BP caused by a 55 W work load was reduced by 5% and 6.5%, respectively ($P<0.05$). Cutaneous perfusion increased by 0.06±0.04 ml/min/100 g in Group I and by 0.11±0.04 ml/min/100 g in Group II (P<0, 05). Hyperaemia recovery time increased significantly by 15.3±4, 6 s in Group I and by 25.2±11.2 s in Group II. Thus, healthy elderly men well tolerate IHT as performed in this investigation. In untrained healthy, elderly men, IHT had greater positive effects on hemodynamics, microvascular endothelial function, and work capacity.

140. CHEMOREFLEX CONTROL OF BREATHING IN HIMALAYAN AND SEA-LEVEL RESIDENTS. M Slessarev[1], E Prisman[1], S Ito[1], R Watson[2], D Jensen[3], D Preiss[1], R Greene[4], T Norboo[5], T Stobdan[5], D Diskit[6], A Norboo[6], M Kunzang[7], JA Fisher[1], J Duffin[1], O Appenzeller[8]. *[1]Departments of Anesthesia and Physiology, University Health Network, University of Toronto[2]University of California, San Diego[33]School of Kinesiology and Health Studies, Clinical Exercise Physiology Lab Respiratory Investigation Unit, Queen's University[4]New Mexico Highlands University[5]Ladakh Institute of Prevention and Ladakh Heart Foundation[6]Sonam Norboo memorial hospital Leh, Ladakh[7] Ladakh autonomous hill development council [8]NMHEMCRF, Albuquerque, NM. Email: marat.slessarev@utoronto.ca*

Introduction: Long-term residents of high altitude exhibit adaptations, such as a blunted ventilatory response to hypoxia. We hypothesized that alterations in the respiratory chemoreflexes indicate the extent of altitude adaptation in a population. Methods: We compared respiratory chemoreflexes of 24 lowlanders (L) with those of 15 native Himalayans living at 4550 m in Ladakh, India, (H) using modified rebreathing tests at hypoxic (50 mmHg) and hyperoxic (150 mmHg) isoxic PO_2 tensions. Results: H had lower ventilatory sensitivities to CO_2 than L at both isoxic tensions (hyperoxic: 2.5 ± 0.4 vs. 4.2 ± 0.3 L/min/mmHg, p= 0.011; hypoxic: 2.8 ± 0.3 vs. 7.1 ± 0.5 L/min/mmHg, $p<0.001$), and the usual increase in ventilatory sensitivity induced by hypoxia in L was absent in H ($p=0.526$). Furthermore, the ventilatory recruitment threshold PcO_2 tensions in H were lower than in L (hyperoxic: 33.3 ± 0.7 vs. 49.0 ± 0.6 mmHg, $p<0.001$; hypoxic: 31.0 ± 1.1 vs. 44.7 ± 0.6 mmHg, $p<0.001$). Both groups had reduced ventilatory recruitment thresholds with hypoxia and there were no differences in the sub-threshold ventilations between L and H at both isoxic tensions. Thus, non-chemoreflex drives to breathe did not differ between groups, nor were they affected by PO_2. The H were older ($p<0.001$), shorter ($p <0.001$) and lighter ($p<0.001$) than L. Conclusion: Lower ventilatory recruitment thresholds and sensitivities to CO_2 seen in H are an index of their altitude adaptation. The lack of an effect of hypoxia on CO_2 sensitivity in H may explain their blunted hypoxic ventilatory responses. Our results therefore suggest that physiological mechanisms other than a hypoxia-induced increase in ventilation may be involved in determining the adaptation of H to life at high altitude. Funded by Isocapnia Research Laboratory and NMHEMC Research Foundation.

141. MAXIMAL AND SUBMAXIMAL EXERCISE PERFORMANCE DURING STAY AT 5533M. Alexander J. Turk[1], Tsogyal D. Latshang[1], Thomas Hess[2], Otto D. Schoch[3], Urs Hefti[4], Tobias Merz[5], Martina M. Bosch[6], Daniel Barthelmes[6], Marco Maggiorini[7], Konrad E. Bloch[1]. *[1]Pulmonary Division, University Hospital, Zurich, Switzerland, [2]Kantonsspital, Winterthur, Switzerland, [3]Kantonsspital, St. Gallen, Switzerland, [4]Kantonsspital, Aarau, Switzerland, [5]Intensive Care, University Hospital, Berne, Switzerland, [6]Opthalmology, University Hospital, Zurich Switzerland, [7]Intensive Care, University Hospital, Zurich, Switzerland. Email: alexander.turk@usz.ch*

Objective: There is little change in maximal oxygen uptake (VO_2max) during acute and sustained exposure to hypoxia but climbers feel they can work harder after acclimatization. We evaluated whether this is related to changes in submaximal exercise performance. Methods: 32 healthy montaineers (mean±SD age 45±11y, 7 women) underwent progressive cardiopulmonary bicycle exercise tests to exhaustion in Zurich (490m), and during ascent to Muztagh Ata, Western China. Tests were performed upon arrival at camp I (5533m, day 1), and 4 to 5 days later (5533m, day 4-5). Maximal performance and submaximal performance at iso-watt corresponding to 75% of maximal work rate (Wmax) at 5533m, day 1, were compared among low and high altitude tests. Results: Wmax (watts) decreased from 356±73 at 490m to 191±49 at 5533m, day 1, and to 193±45 at 5533m, day 4-5. Corresponding values of VO_2max, and arterial oxygen saturation (SpO_2) were 50.7±9.5, 26.3±5.6, 25.3±5.9 ml/min/kg, and 96±3, 71±7, 75±8%, respectively ($P<0.05$ for comparisons of corresponding values at 5533m vs. 490m). At 5533m, day 1, 75% of Wmax was 152±37 watts corresponding to a VO_2 of 23.6±5.4 ml/min/kg, a heart rate of 142±15 beats/min, a minute ventilation of 85.8±21.6 L/min, and an SpO_2 of 68±8%. Iso-watt exercise at 5533m, day 4-5, was associated with a lower VO_2 of 21.6±6.2 ml/min/kg and heart rate of 135±17 beats/min, and a higher SpO_2 of 74±7% ($P<0.05$ vs. corresponding values at 5533m, day 1, all instances), but a similar minute ventilation (83.2±21.0 L/min, P=NS vs. 5533m). Conclusions: A lower heart rate and oxygen uptake, and a higher oxygen saturation for a given submaximal work rate may contribute to the improved tolerance of submaximal exercise during usual activities of mountaineers acclimatizing to high altitude. Grant support: Swiss National Science Foundation.

142. NORMAL COGNITIVE PERFORMANCE IN HIGH-ALTITUDE CLIMBERS ON MUZTAGH ATA: AN ASSESSMENT BY EYE MOVEMENT RECORDINGS AND NEUROPSYCHOLOGICAL TESTS. Merz Tobias[1], Martina Bosch[2], Daniel Barthelmes[3], Timothy Holmes[2], Konrad Bloch[4], Marco Maggiorini[5], Urs Schwarz[6]. *[1]Intensive Care Unit, University Hospital Bern, Switzerland, [2]Ophthalmology Clinic, University Hospital Zurich, Switzerland, [3]Ophthalmology Clinic, University Hospital Bern, Switzerland, [4]Pneumology Clinic, University Hospital Zurich, Switzerland, [5]Intensive Care Unit, University Hospital Zurich, Switzerland, [6]Dept of Neurology, University Hospital Zurich, Switzerland. Email: klinmax@usz.unizh.ch*

Objective: To further investigate the hypothesis that climbing above 5000m leads to various degrees of cognitive impairment, we used a series of neuropsychological tests and - for the first time - compared the results with saccade performance during different stimulus conditions. Eye movement (EM) studies are well-suited to assess the state of the brain: Depending on the stimulus, they distinctly probe various cerebral areas as well as the final neuromuscular pathway and, thus, may be used, for instance, to distinguish between disinterest, attention deficit, fatigue, and cortical injuries. Methods: 32 mountaineers (mean age 43y) participated in a research expedition to Muztagh Ata (7546m). Neuropsychological tests comprised figural fluency, line bisection, cancellations, and a pegboard task. Saccade performance was evaluated in three stimulus conditions: Visually guided pro- (PS) and anti- (AS) saccades, and visuovisual interaction (VVI). For each condition, 128 EMs were sampled during ~3-minute periods using a purpose-built infrared

eye-tracker (ETH, Zurich). Typical saccade parameters (latency, mean-sequence, post-saccadic stability, error rate) were computed off-line. Measurements were taken at 440m, 4497m, 5533m, 6265m, and again at 440m. Results: The neuropsychological test results did not reveal cognitive impairment. Likewise, saccade performances showed no dependence on any altitude related parameter and were well within normal limits: 29756 saccades were measured in 376 individual sessions. At the respective altitudes, the overall mean±SD latencies [ms] for the PS were 185±19, 184±20, 178±21, 174±17, 185±22 (grand mean±SD 185±20); for the AS they were 261±31, 274±36, 265±36, 266±38, 269±38 (267±35); and VVI data yielded 260±20, 278±24, 266±32, 270±24, 284±36 (272±29). Other parameters showed a similarly independent behavior. Conclusions: Our data clearly showed that well acclimatized climbers do not seem to suffer from significant cognitive deficits even at very high altitudes. Furthermore, we demonstrated that the investigation of EMs is feasible during high altitude expeditions. Support: Swiss National Science Foundation.

143. SPATIAL ANALYSIS FOR STUDYING THE RELATIONSHIP OF ALTITUDE AND HEALTH OUTCOMES. D Thomas[2], B Honigman[1], S Niermeyer[1,3], M Egbert[4]. *[1]Altitude Research Center, Division of Emergency Medicine, [2]Department of Geography, [3]Department of Pediatrics, University of Colorado at Denver and Health Sciences Center, [4]Colorado Department of Public Health and Environment. Email: Deborah.Thomas@cudenver.edu*

The relationship between altitude and its impact on common clinical diseases and aging is not well understood. Most previous investigations have examined the effects of extreme altitudes rather than elevations where people typically live. Colorado is an obvious place to study health effects of moderate altitude. Over 14% of the total population (584, 000 people) live at/above 7000 feet and over 25 million tourists visit annually. This exceeds any other U.S. state. Traditional epidemiologic methods demonstrate some interesting health trends and demographics in Colorado. Of the 10 U.S. counties ranked highest for life expectancy, 8 are in Colorado. Our state has lower mortality rates for stroke, heart disease and cancer; yet its rate for certain respiratory diseases and Alzheimer's are significantly higher than other parts of the country. The Altitude Research Center at the University Of Colorado School Of Medicine has begun to integrate spatial analyses in order to more specifically identify the association between disease and altitude. By utilization of spatial analysis/GIS technologies as a methodological tool to arrive at an increased understanding of the role of altitude, we have shown that it has an impact on birth weight, RSV in children, and multiple sclerosis. The mechanism of this association is not understood, but by combining existing medical databases with GIS methodology to examine the impact of moderate altitude on clinical diseases we can gather data that will further our ability to identify the relationship between altitude and common clinical conditions.

144. DETERMINANTS OF BLOOD OXYGENATION DURING PREGNANCY IN ANDEAN AND EUROPEAN RESIDENTS OF HIGH ALTITUDE. M Vargas[1], E Vargas[1], CG Julian[2,3], A Rodriguez[1], JA Armaza[1], W Tellez[1], S Niermeyer[2], MJ Wilson[2,3], E Parra[4], M Shriver[4], LG Moore[2,3]. *[1]Instituto Boliviano de Biología de Altura, La Paz, BO[2]Altitude Research Center, University of Colorado at Denver and Health Sciences Center, Denver, USA[3]Dept of Health/Behavioral Sciences, University of Colorado at Denver and Health Sciences Center, Denver, USA[4]Anthropological Genetics Lab, Pennsylvania State University, State College, USA*

Objective: To determine whether greater maternal arterial oxygenation was responsible for the heavier birth weights seen in long- vs. short-resident high-altitude populations. Methods: Ventilatory and hematological studies were conducted in 42 Andean and 26 European residents of La Paz, Bolivia (3600 m) serially during pregnancy and 4 mo postpartum. Results: Pregnancy

raised hypoxic ventilatory sensitivity 3-fold, ventilation (VE), and arterial O_2 saturation (SaO_2) in both groups. Women with greater Andean genetic ancestry had higher respiratory frequency and lower tidal volume. Pregnancy increased total and plasma volume ~40% without changing red cell mass. The hemoglobin decline was compensated for by higher VE and SaO_2 such that arterial O_2 content (CaO_2) was maintained at nonpregnant levels in both groups. After adjusting for variation in gestational age, maternal height and parity, Andeans weighed 209 gm more than Europeans. Babies with greater ponderal indices were born to Andean women with higher VE at pregnancy weeks 20, 30 and 36 ($R2 = 0.27$, 0.30 and 0.25 respectively, all $p<0.05$). Week 20 VE also correlated with infant birth weight ($R2 = 0.15$, $p<0.05$) in the Andean, but not European women. Conclusions: We concluded that while maternal VE was important, some factor other than higher CaO_2 was responsible for protecting Andeans from altitude-associated reductions in fetal growth. (NIH-TW001188, HL60131 and HL079647; AHA and predoctoral fellowships).

145. DOES CHRONIC MOUNTAIN SICKNESS (CMS) HAVE PERINATAL ORIGINS?
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CMS is a potentially fatal but poorly understood disorder characterized by excessive erythrocytosis (EE), circulatory and CNS dysfunction affecting ~10% of adult male residents of high altitude. Objectives: We asked if gestation and birth in a hypoxic environment increased susceptibility to CMS by assessing birth weight and related variables in young persons without the confounding effects of advancing age or lung disease. Methods: Sixty-two (62) young males (15-35 yrs) with EE were identified (hemoglobin>2 STD dev above the mean) from community surveys of 8200, 10-60 yr old residents of 3200-4850 m, for an estimated EE prevalence of 7.5% in 15-35 yr old men. Twelve (12) were available for study. Results: All 12 were hypoxic in utero or neonatally as demonstrated by low birth weight (2571+243 gm), prematurity (8/12) or diagnosis of neonatal hypoxia (11/12). Half their mothers (6/12) had mild or severe (n=2) preeclampsia. All were lifelong high-altitude residents, of normal BMI but low SaO_2 (87.5+1.0%) due to hypoventilation ($PETCO_2$=35+1 mmHg). FVC and FEV1.0. were normal but FEF50-75% diminished, suggesting air-trapping. Most (8/10) demonstrated electrocardiographic evidence of pulmonary hypertension and RVH.

146. GREATER UTERINE ARTERY BLOOD FLOW DURING HIGH-ALTITUDE PREGNANCY IN INDIGENOUS (ANDEAN) THAN FOREIGN (EUROPEAN) WOMEN.
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Objective: To determine if uterine artery (UA) blood flow raised uteroplacental O_2 delivery to a greater extent in multigenerational (Andean) vs. shorter-term (European) residents of high altitude (3600 m). Methods: Doppler ultrasound studies were conducted during pregnancy and at 4 mo postpartum in 42 Andean and 26 European residents of 3600 m. Results: Pregnancy increased UA diameter to a greater extent in Andean than European women, raising UA blood flow and O2 delivery 6-fold in the Andeans vs. 3-fold in the Europeans. The Andeans had greater com-

mon iliac (CI) and external iliac (EI) flows in combination with greater UA/EI and lower EI/CI, suggesting greater redistribution of lower extremity flow to favor the UA than in the European subjects. After adjusting for known covariates, fetal biometry was greater at weeks 20 and 30 and birth weights 209 gm heavier in the Andeans vs. European deliveries. Lower UA resistance index (RI) correlated with larger fetal abdominal circumference at 36 wk in the Andeans alone. Conclusions: Andeans are protected from altitude-associated reduction in fetal growth by being able to maintain a normal pregnancy-associated increase in UA blood flow, perhaps as the result of genetic factors influencing maternal vascular adjustment to pregnancy. (NIH-TW001188, HL60131 and HL079647; AHA and predoctoral fellowships).

147. FINDING GENE CANDIDATES FOR NATURAL SELECTION IN HIGH-ALTITUDE PREGNANCY. Megan J. Wilson[1,2], Abigail Bigham[3], Mark Shriver[3], Colleen G. Julian[1,2], Enrique Vargas[4], Lorna G. Moore[1,2]. *[1]Altitude Research Center, University of Colorado at Denver and Health Sciences Center, [2]Dept of Health and Behavioral Sciences, University of Colorado at Denver and Health Sciences Center, [3]Anthropological Genetics Lab, Pennsylvania State University, [4]Instituto Boliviano de Biología de Altura, La Paz, BO. Email: megan.wilson@uchsc.edu*

Fetal growth is slowed at high altitude (>2500 m) and preeclampsia more common, both of which decrease birth weight and raise perinatal morbidity/mortality. We considered that (1) natural selection at high altitude would have targeted genetic factors contributing to these disorders and (2) the genes involved likely included those in the hypoxia-inducible factor (HIF) pathway. OBJECTIVES: To test whether genetic adaptations in HIF-regulatory or targeted genes had been targeted, we compared single nucleotide polymorphisms (SNPs) in Andeans vs. low-altitude control populations (low-altitude Amerindians and Han Chinese). METHODS: In 50 multigenerational high-altitude Andeans, 593 SNPs were evaluated in 59 HIF-pathway genes. Results were analyzed using locus specific branch lengths (LSBL) and the natural log of the ratio of heterozygosity (lnRH) with a sliding windows approach, in which reduced heterozygosity suggests directional selection. Regions that fell in the 0.05 tail of respective negative (lnRH) or positive (LSBL) empirical distributions were considered significant. RESULTS: LSBL and lnRH assessments converged in identifying three gene regions as differing between Andeans and controls: inducible nitric oxide synthase, tenascin-C, and the mammalian target of rapamycin (syn. AMPKα-1). Each is involved in pregnancy and hypoxia-related vascular remodeling. LnRH results identified 2804 regions that differed between Andeans and controls, for which a high (31%) fraction were within HIF-related gene regions. CONCLUSIONS: The high proportion of HIF-related SNPs within low heterozygosity regions supports the involvement of HIF pathway genes in hypoxia-related adaptations in the Andean population. The functional roles of the three candidate genes suggest the mechanism of Andean adaptation during pregnancy targets vascular remodeling. (NIH HL60131, TW 01188, HL07171; NSF Graduate Research Fellowship).

148. INTIMA-MEDIA THICKNESS IN LADAKH HIGHLANDERS. R.R. Watson[1], E.R. Greene[2], E. Prisman[3], M. Slessarev[3], S Ito[3], T Norboo[4], T Stobdan[4], D Diskit[5], A Norboo[5], M Kunzang[6], J.A. Fisher[3], O. Appenzeller[7]. *[1]USCD, La Jolla, CA, [2]NMHU, Las Vegas, NM, [3]U of Toronto, Toronto Gen Hosp, Toronto, Canada, [4]LIP/LHF, Leh, Ladakh, India, [5]S Norboo Mem Hosp, Leh, Ladakh, India, [6]LAHDC, Leh, Ladakh, India, [7]NMHEMCRF, Albuquerque, NM. Email: joe.fisher@utoronto.ca*

Background: Native highlanders from Ladakh, India (4550 m) (H) have a high animal fat consumption and a high prevalence of hypertension, both major risk factors for atherosclerosis. On the other hand they also have normal BMIs and high daily aerobic work loads, both considered protective against atherosclerosis. Noninvasive ultrasound permits measurements of intima-me-

dia thickness (IMT), a surrogate for subclinical atherosclerosis. Objective: To compare common carotid artery IMT in 96 (20 female) H to those measured in age-matched healthy people from industrialized normobaric areas (N). Results: Data from H are mean (SD): age 44 (15) years; BMI 25 (5); MAP 96 (16) mmHg; HR 76 (11) beats per minute; SaO_2 84 (4) %; IMT 0.52 (0.12) mm. The linear regression equation relating age to IMT was IMT = 0.006(Age) + 0.24 ($r=0.74$, $p<0.05$). IMT in age-matched N ranged from 0.60- 1.10 mm. When graphing IMT against age in N, 95% confidence limits for slopes were 0.009-0.016 and for intercepts were 0.12-0.19. Conclusion: This field study suggests that compared to industrialized, normobaric populations, IMT is attenuated in Ladakh highlanders. This reduction occurs in spite of risk factors which increase IMT at sea level, and may be related to their aerobic load and genetics. Our findings are consistent with the low prevalence of cardiovascular diseases in this cohort. Funded by NMHEMC Research Foundation.

149. ADAPTATION TO ALTITUDE AS A VEHICLE FOR EXPERIENTIAL LEARNING OF CARDIOPULMONARY PHYSIOLOGY BY UNIVERSITY UNDERGRADUATES. David Weigle[1], Amelia Buben[2], Caitlin Burke[2], Nels Carroll[2], Brett Cook[2], Benjamin Davis[2], Rian Fisher[2], Timothy Freeman[2], Stephen Gibbons[2], Hale Hansen[2], Kimberly Heys[2], Brittany Hopkins[2], Brittany Jordan[2], Katherine McElwain[2], Katherine Reinhart[2], Charles Robbins[2]. *[1]Department of Medicine, University of Washington, [2]College of Arts and Sciences, University of Washington. Email: weigle@u.washington.edu*

An experiential learning activity is described in which 19 university undergraduates explored physiological adaptation to high altitude by formulating hypotheses and making experimental observations on each other. Following 2 weeks of didactic sessions and baseline data collection at sea level, the group ascended rapidly to the Barcroft Laboratory of the White Mountain Research Station at 12,500 feet elevation. Here, teams of 3-4 students each measured maximal rate of oxygen uptake, cognitive function, hand and foot volume changes, reticulocyte count and hematocrit, urinary pH and 24-hour urine volume, athletic performance, and nocturnal blood oxygen saturation. Their data allowed the students to quantify the effect of altitude on the oxygen cascade and to demonstrate the following altitude-related changes: i. impaired performance on selected cognitive function tests, ii. mild peripheral edema, iii. rapid reticulocytosis, iv. urinary alkalinization and diuresis, v. impaired aerobic but not anaerobic exercise performance, vi. inverse relationship between blood oxygen saturation and resting heart rate, and vii. regular periodic nocturnal oxygen desaturation events accompanied by heart rate accelerations. The students learned and applied basic statistical techniques to analyze their data, and each team wrote up its results in the format of a scientific paper. The students were uniformly enthusiastic about the use of self-directed experimentation to explore the physiology of altitude adaptation, the submission of a manuscript describing their experience to a peer reviewed journal, and the invitation to present their results at the biennial International Hypoxia Symposium.

150. CONTRIBUTION OF RESPIRATORY CHEMOSENSITIVIES ON BREATH HOLDING PERFORMANCE. Mari Yokoi[1], Chikako Yoshino[2], Atsuko Masuda[1], Shigeru Masuyama[1]. *[1]Faculty of Health Science, Ryotokuji University, [2]Chiba College of Allied Medical. Email: yokoi@ryotokuji-u.ac.jp*

It has been thought that ventilatory response to hypoxia and hypercapnia play an important role to determine breath holding time (BHT). The aim of present study was to examine which type of hypoxic ventilatory response (HVR) or hypercapnic ventilatory response (HCVR) has stronger contributor on BH performance. In 15 healthy subjects, BHT, the lowest SpO_2, the lowest $PETO_2$ and highest $PETCO_2$ were measured at breaking point (BP) of BH trials with or without oxygen inhalation. BH trials started from three different lung volumes, i.e., total lung

capacity (TLC), functional residual capacity (FRC) and residual volume (RV). The data were compared with their isocapnic progressive HVR and HCVR by Read's method1). HVR showed significant negative correlation with $PETO_2$ and SpO_2 at BP as well as positive correlation with BHT. HCVR had not clear relationship with any parameters. We conclude that HVR, but not HCVR, is a strong contributor to BH performance and that alveolar PO_2 is a key determinant to BHT other than PCO_2.

SUBJECT INDEX

A

B

C

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

U

V

W, X, Y and Z