Chapter 1

Review of Literature
1.1 HIGH ALTITUDE ENVIRONMENT

The medical definition of high altitude (HA) is an elevation of 2,700–5,500 m above sea level while extreme altitudes have an elevation beyond 5,500 m. At sea level, the concentration of oxygen is about 21% while the barometric pressure averages 760 mmHg. Atmospheric pressure progressively decreases with increase in altitude. Although the percentage of oxygen in inspired air is constant at different altitudes, the fall in atmospheric pressure at HA decreases the partial pressure of inspired oxygen and hence the driving pressure for gas exchange in the lungs (Fig. 1.1). The lower air pressure makes it difficult for $O_2$ to diffuse into the vascular system resulting in $O_2$ deprivation or hypoxia which is also known as hypobaric hypoxia (Peacock 1998; Sarkar et al. 2003). HA primarily affects the human body because of $O_2$ deprivation. Other factors, such as severe cold, high winds, and intense solar radiation, may be present but can be overcome by appropriate protection. Hypoxia is inevitable unless it is relieved by supplementary oxygen or unless the person is placed in a container at increased pressure, such as a Gamow bag (West 2004).

1.2 ACCLIMATIZATION TO HIGH ALTITUDE

The adaptive changes collectively known as acclimatization greatly improve the tolerance of human beings to HA (West 2004). The most important feature of acclimatization is the increase in respiratory drive or hyperventilation, brought about by hypoxic stimulation of the peripheral chemoreceptors, mainly the carotid body type 1 glomus cells, which sense the low $P_O_2$ in the arterial blood. It has been suggested that hypoxia stimulates the carotid bodies to release excitatory neurotransmitter(s) that act on nearby afferent nerve endings leading to an increase in sensory discharge. Hypoxia increases the release of dopamine and synthesis as well as the activity of tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine biosynthesis. The mRNA levels of TH increases within 1 h of exposure to moderate sustained hypoxia, peaks at 6 h and remains elevated for up to 48 h (Prabhakar 2000; Czyzyk-Krzeska 1997). Increases in alveolar $P_O_2$ with hyperventilation facilitate $O_2$ loading in the lungs and provide the rapid first line of defense against reduced ambient $P_O_2$ at HA.
Fig. 1.1: Relation between Altitude and Inspired Oxygen Pressure
Cardiac output increases following exposure to HA. The heart pumps blood faster, so that oxygen is transported from the alveoli through the circulatory system to tissues at a faster rate hence rise in the blood pressure. After few weeks at HA, the cardiac output for a given work rate is the same as at sea level but the heart rate remains high. Thus, the stroke volume (determined by cardiac output divided by heart rate) is reduced.

One more aspect of altitude acclimatization is the increase in red cell numbers per unit volume and the increase in hemoglobin (Hb) concentration. The cool and dry ambient air in mountainous regions results in considerable body water evaporation. At HA acute hypoxic conditions lead within hours to a 10% plasma volume decrease, while red cell volume remains unaffected resulting in increased hematocrit and Hb concentration (Sawka et al. 2000). Hypoxia also induces the release of erythropoietin (EPO), which stimulates the bone marrow to increase the red cell output (West 2004). EPO being the first discovered example of hypoxia-dependent gene expression, results in the polycythaemic response to hypoxia, which not only leads to increased hematocrit but increased blood viscosity as well. This in turn increases oxygen delivery and is one of the most fundamental adaptive responses to altitude (Ge et al. 2002). Short or prolonged residency at HA is associated with increased secretion of EPO and is markedly enhanced in HAPE patients (Milledge and Cotes 1985; Basu et al. 2007). Oxygen carried by the arterial Hb is measured and reported as the arterial oxygen saturation (SaO$_2$). At HA, exposure to ambient hypoxia causes reduced levels of SaO$_2$. The O$_2$ content of blood is the product of capacity and saturation (SaO$_2$) plus the dissolved O$_2$. Thus, increase in concentration of Hb compensates for a reduction in arterial SaO$_2$.

Humans also experience decrease in oxygen affinity of blood that facilitates gas delivery to tissues at the expense of uptake in the lungs. This functional property of Hb is regulated by 2,3-Bisphosphoglycerate (2,3-BPG), a small soluble organic phosphate compound present in high concentration inside red blood cells (~5 mM) where it binds to the deoxy form of Hb and decreases its oxygen affinity. Consequently, 2,3-BPG plays a key role in facilitating the supply of oxygen to tissues (Rose and Liebowitz 1970). During acclimatization, the numbers of capillaries are also increased; new capillaries
facilitate diffusion required for oxygen to reach cells. These mechanisms ensure increased oxygen delivery to cells and efficient use of available oxygen.

A decrease in CO$_2$ level with hyperventilation increases the pH due to loss of carbonic acid, and body fluids become more alkaline. However, after a day or so, the pH of the cerebrospinal fluid changes toward normal by movement of bicarbonate out of the cerebrospinal fluid, and after 2 or 3 days the pH of the arterial blood moves toward normal by renal excretion of bicarbonate. The initial alkalosis in both the cerebrospinal fluid and the blood tends to inhibit hyperventilation through the action of both the central chemoreceptors in the brainstem and the peripheral chemoreceptors in the carotid and aortic bodies. The sensitivity of the carotid body to hypoxia also increases during prolonged exposure to HA (West 2004).

The transcription factor, hypoxia inducible factor (HIF1) is pivotal in the cellular response to the stress of hypoxia as it functions as a global regulator of O$_2$ homeostasis facilitating both O$_2$ delivery and adaptation to O$_2$ deprivation (Semenza 1999). HIF1, a basic-helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) transcription factor, consists of HIF1A and HIF1B subunits (Wang et al. 1995). Transcription of “hypoxia-sensitive” genes is promoted by binding of the 3’ transcriptional activation sequence called the hypoxia-responsive element (HRE) by HIF1. Decreased oxygen concentration increases HIF1A stability through post-translational modifications (Huang et al. 1998), which induces several target genes (Fig. 1.2) involved in erythropoiesis, glycolysis, glucose transport, vasodilatation and angiogenesis (Ryan et al. 1998; Ratcliffe 2002; Pugh and Ratcliffe 2003; Palmer et al. 1998; Iyer et al. 1998; Carmeliet et al. 1998). The product of these genes either increases oxygen delivery or allows metabolic adaptation to reduced oxygen availability. As HIF1A is an essential mediator of the adaptive response to hypoxia, we cloned and sequenced HIF1A cDNA from yak, a mammal native to the Himalayas that has successfully adapted over many generations to the chronic hypoxia of HA. HIF1A cDNA revealed the evolutionary conservation through multiple sequence alignment (Dolt et al. 2007b).
Fig. 1.2: The Signaling Pathway of Hypoxia Inducible Factor 1 Alpha
1.3 ADAPTATION

‘Adaptation’ is a process by which organisms respond to long term environmental stress by permanent genetic change (Baker 1969). It is a fitness-enhancing trait, which was produced by selection for its current fitness benefits. In other words, it is a performance of function in the context of interaction between organism and its external environment such that survivorship is maintained between generations. Most researchers in comparative biology accept that a species dealing with hypoxia, or any other selectively relevant factor, depends upon the time available for orchestrating the response. Usually, the timeline for response is divided into three categories: acute, acclimatory, genetic or phylogenetic (Hochachka et al. 1998). A physiological system may change randomly due to genetic drift. Recent studies addressed the question as to whether genetic adaptation to HA has occurred. Common to these studies are the use of oxygen transport system and the passage of time as organizing principles, and the recognition of the multifaceted ways in which genetic factors can influence physiological processes (Moore et al. 2000). These studies differ in terms of approach and sources of evidence for judging the duration of HA residence. Migrant, family set and admixture study designs have been used for comparisons within populations. These collectively demonstrate the existence of genetic influences on physiological characteristics.

Natural selection, especially on traits in the pathway of oxygen uptake and utilization may have driven HA settlers to adapt to hypoxic environment. There are at present three major HA regions: Himalayas and Tibetan Plateau (Tibetans, Ladakhi and Sherpas), Andes (Quechua, Ayamara, Inca), and Africa (Ethiopians) having distinctive pattern of adaptation (Rupert and Hochachka 2001; Beall et al. 2002; Beall et al. 2001). Another HA settlement of interest from Asia is the Kyrgyz population settled in the Tien-Shan and Pamir Mountains in central Asia. These regions have been settled for different lengths of time and have also experienced a continuous inflow of sojourners and migrants, which affords the possibility of comparing different population groups with different time depths of exposure to hypobaric hypoxia. According to the literature, the high mountains have been occupied for
several thousand years; for example the Andean Plateau was occupied ~11,000 years ago and the Tibetan Plateau ~22,000 years ago; however, the settlement time is highly debated by experts in the field (Beall 2007; Aldenderfer 2003). These populations may have different and distinctive patterns of adaptation to the same environmental stressor, perhaps attributed to different time intervals spent at altitude (Rupert and Hochachka 2001; Beall et al. 2002; Beall et al. 2001).

If one sees the adaptive trend, the Tibetans and Andeans have adapted differently. While the Hb concentration is found lower in Himalayan than Andean highlanders (Beall et al. 1998; Beall 2007), the Tibetans are similar to sojourners with higher hypoxic ventilatory response (HVR) compared to Andeans (Brutsaert 2007; Zhuang et al. 1993). Chronic mountain sickness (CMS) is rare in the Himalayas while common in Andes (Leon-Velarde et al. 2003). The genetic differences between populations are relatively small compared with those within populations (Tang et al. 2005; Rosenberg et al. 2002; Nei and Roychoudhury 1974; Cavalli-Sforza and Feldman 2003). Nevertheless, Tibetan versus Andean differences could have a genetic component, although this issue is yet to be resolved.

1.4 FAILURE OF ACCLIMATIZATION: HIGH ALTITUDE ILLNESS

People are traveling in increasing numbers to the mountains of North America, Europe, Asia, and South America for a variety of business, lifestyle-oriented and recreational reasons such as skiing, hiking, mountain climbing, and other tourist activities. The soldiers posted at HA are exposed to the harsh conditions characteristic of the HA terrain, which affects the conduct of military operations. The military has very strong reasons to carry out HA research and training because of the Indian Army's experience in the mountains of Kashmir. As increasing numbers of sojourners are visiting the mountains, HA illness is becoming a pathological phenomenon about which healthcare providers should have greater awareness. HA illness is the collective term for the syndromes that can affect unacclimatised travelers shortly after ascent to HA. The term encompasses the syndromes of acute mountain sickness (AMS), high altitude cerebral edema (HACE), high altitude pulmonary edema (HAPE) and CMS. HACE and HAPE
occur much less frequently than AMS, but are potentially fatal (Monge and Whittenbury 1976; Hackett and Roach 2001; Basnyat and Murdoch 2003).

1.4.1 Acute Mountain Sickness

AMS is an illness that can affect mountain climbers, hikers, skiers, or travelers who climb too fast. It usually occurs when people rapidly reach a HA (typically above 8,000 feet or 2,400 meters). The incidence of AMS depends upon the rate of ascent and the height reached. AMS is characterized by non-specific symptoms and a scarcity of physical findings. The main symptoms are headache, anorexia, nausea, vomiting, fatigue, dizziness, and sleep disturbance, but not all need to be present. Symptoms of AMS typically appear 6–12 h after arrival at HA.

Pathophysiology: The exact process of AMS is unknown. Roach and Hackett had proposed a model to explain the pathophysiology of AMS (Roach and Hackett 2001). In this model, the initial insult is hypoxemia. Hypoxemia is translated through a series of cellular, molecular and physiological signals ultimately to cause brain swelling due to cerebral edema and elevated cerebral blood volume (CBV). Hypoxemia stimulates cellular and molecular responses that may alter endothelial permeability (vascular endothelial growth factor) or provide cellular protection against oxygen-derived free radical damage to the endothelium. Hypoxemia is also implicated in upregulation of inducible nitric oxide synthase, and its product nitric oxide (NO) has been implicated in the pathophysiology of headache and blood–brain barrier (BBB) permeability. Through peripheral chemoreceptor activation, hypoxemia can elevate circulating arginine vasopressin levels which, in turn, cause anti-diuresis and increased extracellular water levels. The sum of these peripheral responses to hypoxia arrives at the brain and influences BBB permeability, cerebral edema and CBV. These changes cause elevated intracranial pressure in those with a cerebrospinal capacitance that cannot buffer the swelling brain. Intracranial pressure causes the symptoms of AMS via compression of the brain. The interplay between brain water content, brain blood volume and intracranial dynamics ultimately determines who develops AMS.
1.4.2 Chronic Mountain Sickness

In 1925, Carlos Monge M. reported a case of polycythemia in a patient from Cerro de Pasco (4300 m) to the Peruvian Academy of Medicine. In 1928, he reported a series of such patients with red cell counts significantly higher than normally found at altitude. This condition of CMS has come to be known also as Monge’s disease (Monge 1942; Monge and Whittembury 1976). Patients typically have rather vague neuropsychological complaints including headache, dizziness, somnolence, fatigue, difficulty in concentration and loss of mental acuity. There may also be irritability, depression, and even hallucinations. Dyspnoea on exertion is not commonly complained of, but poor exercise tolerance is common and patients may gain weight. The characteristic feature of the disease is that the symptoms disappear on descending to sea level, only to reappear on return to altitude. The red cell count, Hb concentration and packed cell volume (PCV) are raised; values as high as 28 g Hb dl\(^{-1}\) and a PCV of up to 83% have been recorded. Blood gases, compared with healthy controls at the same altitude, show a higher PaCO\(_2\) and lower PaO\(_2\) and oxygen saturation. The very high haematocrit increases the viscosity of the blood enormously. The systemic blood pressure may be moderately elevated and the pulmonary artery pressure (PAP) is significantly higher than healthy HA residents (Ward et al. 2000).

**Pathophysiology:** The physiopathology of CMS has been attributed to the following sequence: blunted respiratory response to hypoxia, hypoventilation, excessive hypoxemia and excessive erythropoiesis. Altitude hypoxia and hypoventilation will result in low PaO\(_2\). This hypoventilatory response may be due to a low HVR to hypoxic depression of ventilation or some unknown cause. If lung function is also reduced by lung or chest wall disease, this will reduce PaO\(_2\) still further. Aging results in both reduced lung function and reduced HVR, especially in a life time spent at HA, thus further reducing PaO\(_2\). The low PaO\(_2\) results in low SaO\(_2\). It also stimulates the secretion of EPO, and hence an increase in PCV. The rise in PCV causes a rise in blood viscosity and fall in cerebral blood flow, which, with a low SaO\(_2\), results in chronic severe cerebral hypoxia and symptoms of CMS (Ward et al. 2000).
1.4.3 High Altitude Pulmonary Edema

HAPE is noncardiogenic pulmonary edema that usually occurs at altitudes above 3,000 m in rapidly ascending nonacclimatized individuals within the first 2–5 days after arrival. It may also occur in HA dwellers who return from sojourns at low altitude or move up at further heights. HAPE was independently described in the Andes in the 1930s by Alberto Hurtado and published in the Peruvian medical literature. The first cases of HAPE in unacclimatized lowlanders climbing to HA were reported from the Rocky Mountains (Bartsch et al. 2005; Houston 1960). The prevalence of HAPE depends on the degree of susceptibility, the rate of ascent, and the final altitude. The first symptoms of HAPE are generally dyspnoea on exertion and reduced exercise tolerance greater than expected for the altitude. Cough, dry and annoying at first, becomes productive later in the illness with bloodstained sputum. Physical findings may be initially subtle. Tachypnoea and tachycardia are present at rest as the illness progresses, and fever is common, although rarely exceeding 38.3°C. Crackles are evident on chest auscultation. HAPE is frequently accompanied by signs of HACE (Basnyat and Murdoch 2003).

Pathophysiology: HAPE is characterised by exaggerated pulmonary hypertension leading to vascular leakage through overperfusion, stress failure, or both. The exact mechanism that causes the accentuated hypoxic pulmonary vasoconstriction is unclear. Undoubtedly, several factors combine to render an individual susceptible to HAPE (Fig. 1.3). Venoconstriction causes an increase in both alveolar-capillary pressure and vascular-fluid shear stress. Uneven regional blood flow may cause flooding of some capillaries. Capillary permeability may increase, as reactive oxygen species (ROS), chemical mediators of inflammation, and such growth factors as vascular endothelial growth factor (VEGF), interleukin-1, and tumor necrosis factor α (TNFα) are released from pulmonary structural cells and alveolar macrophages, as well as from neutrophils and platelets that become trapped in the pulmonary microvasculature. Ion channels, such as the epithelial sodium channel, and the Na⁺/K⁺-ATPase, which act as “defenders” of dry alveolar space, are challenged by hypoxia.
Fig. 1.3: The Complex Pathobiology of High Altitude Pulmonary Edema
Gene polymorphisms that confer differences in the activities of key enzymes may play a part in the pathogenesis of HAPE (Voelkel 2002; Basnyat and Murdoch 2003).

1.4.4 High Altitude Cerebral Edema

HACE is widely viewed as the end stage of AMS, and is normally preceded by symptoms of AMS. HACE is characterized by ataxia and altered consciousness, which may progress to coma and death due to brain herniation. People with concomitant HAPE may progress very rapidly from AMS to HACE. Clinical examination may reveal papilloedema, ataxia, retinal haemorrhages, and, occasionally, focal neurological deficits.

**Pathophysiology:** Fluid may be pulled into the cerebral tissue by virtue of increased extravascular osmotic pressure or pushed from the intravascular to the extravascular cerebral space because of increased vascular hydrostatic pressure. These two respective modes are commonly referred to as cytotoxic and vasogenic edema. Cytotoxic edema may produce HACE as a consequence of hypoxia-induced failure of cellular ion pumps, increased intracellular osmolarity, and consequent cell swelling. However, though such changes may contribute during the late stages of illness, it is unlikely that early symptoms could be associated with hypoxemia sufficient to impair cell homeostasis. Thus, current thinking is that brain edema is more likely of vasogenic than cytotoxic origin. Neuroimaging studies in persons with HACE have demonstrated the presence of vasogenic edema, supporting the etiology of increased intracranial pressure with HACE. Hemodynamic factors such as impaired cerebral autoregulation, sustained vasodilation, and elevated cerebral capillary pressure are thought to contribute to edema formation. Other contributing factors may be mediators such as bradykinin, inducible nitric oxide synthase, and VEGF, all of which have been implicated in hypoxia-induced biochemical alteration of the BBB (Rodway et al. 2003; Hackett et al. 1998).

1.5 ACUTE HYPOBARIC HYPOXIA EXPOSURE IN ANIMALS AND HUMANS

Ladakh is a sparsely populated area of Indian Himalaya lying at 3–4,500 m altitude mainly consisting of arid desert. Located with the Karakoram
to the northwest, the Himalayas in the southwest, and the Trans-Himalayas at its core, this region has permanent villages at an elevation as high as 4,650m. Sojourners who visit this region for various reasons like officials, tourists and laborers may experience initial discomfort, which sometimes culminates into HA disorders. HA natives who have occupied the highlands for thousands of years do not suffer from such disorders and carry out the routine activities and other difficult tasks with ease. It is believed that how a species deals with hypoxia, depends upon the time available for orchestrating the response. Usually the timeline for response is divided into three categories: acute, acclimatory, and genetic or phylogenetic. Acute exposure to HA invoke hypoxia defenses in humans initiated by several oxygen sensing, signal transduction pathways. At the base of the acute hypoxia response systems are tissue-specific O$_2$ sensing systems which in turn regulate the synthesis of HIF1 (Hochachka 1998; Hochachka et al. 1999). The advent of DNA microarray technology has greatly expanded the ability of investigators to identify novel hypoxia-responsive genes in various human cell lines subjected to varying periods and degree of hypoxia (Burke et al. 2003; Ning et al. 2004; Chi et al. 2006; Sonna et al. 2003; Jiang et al. 2002; Mense et al. 2006) and the transcriptional response to hypoxia varied among various human cell lines.

In 1946, the U.S. navy conducted a 5 week test called as ‘Operation Everest’ to study the physiological changes at altitude where four subjects were simulated up to 29,000 ft (Graybiel et al. 1950). In 1985, ‘Operation Everest II’ was designed to examine the effects of hypobaric hypoxia under the controlled conditions of a decompression chamber in which human volunteers lived for 40 days while the pressure within the chamber was gradually reduced, simulating an ascent of Mt Everest (8,848 m) (Houston et al. 1987). A number of studies were carried out during Operation Everest II, few of which reported metabolic and hormonal responses to incremental exercise (Young et al. 1992), alterations in immune system (Meehan et al. 1988), oxygen transport and cardiovascular function (Sutton et al. 1992) plasma lipid and hormonal responses (Young et al. 1989) and neuromuscular function (Garner et al. 1990) at extreme altitude. Operation Everest III (Comex '97) was the third simulated climb of Mt Everest in a hypobaric chamber which examined the lipid
peroxidation and antioxidant defense system (Joanny et al. 2001), energy and water balance (Westerterp et al. 2000), cardiac function (Boussuges et al. 2000), appetite (Westerterp-Plantenga et al. 1999) etc. in subjects simulated to Mt Everest for 32 days.

However, a big challenge to human biology investigators when trying to determine the effects of extreme environmental stress in different tissues of humans is that they cannot be subjected to life-threatening environments and furthermore, usually only peripheral blood cells are available for study, and individual subjects exhibit considerable genetic differences. However, instead we can use inbred strains of animals such as mice which will provide equally consistent results as they will be essentially genetically identical, moreover, we can study the response in different tissues as well. Normal tissue oxygenation lies in a range that is optimized for the individual tissue. For example, while normoxic lung may be near atmospheric levels of oxygen (21% O$_2$, or 158mm Hg partial pressure), tissues such as the liver, cartilage, bone marrow or lens of eye commonly exist with much lower oxygenation levels, in the 2–8% range (Papandreou et al. 2005). Hence, tissues will vary considerably in their sensitivity to hypoxia.

Liver

Liver, the largest metabolic organ in the body performs a number of important and complex biological functions that are essential for survival. It has an important role in metabolism of carbohydrates, proteins, lipids, drugs etc. Previous studies have analyzed the effect of hypoxia on different hepatocellular carcinoma cell lines (Sonna et al. 2003; Vengellur et al. 2005). However, the molecular details of acute hypobaric hypoxia (AHH) induced transcriptional changes in murine liver were investigated first by us. Several of the genes involved in sterol metabolism were found to be down regulated more than two fold (Fig. 1.4) suggesting that hypoxia suppresses sterol biosynthesis in the liver (Dolt et al. 2007a). Furthermore, the study highlighted the significance of Sterol Regulatory Element Binding Protein (SREBP) in the regulation of sterol metabolism under the acute hypoxic response.
Fig. 1.4: Transcriptional Downregulation of Sterol Metabolism Genes in Murine Liver Exposed to 10 h of AHH
(Dolt KS et al, Biochem Biophys Res Commun. 2007;354:148-53)
Heart

At HA there is initially an increase in cardiac output in relation to physical work but later this settles to sea level values. At all times there is increased heart rate and decreased stroke volume for a given level of work, though the maximum obtainable heart rate falls as higher altitudes are reached (Peacock 1998). Since the mammalian heart is an obligate aerobic organ, a constant supply of oxygen is indispensable to sustain cardiac function and viability. Oxygen participates in the generation of nitric oxide, which plays a critical role in determining vascular tone, cardiac contractility, and a variety of additional parameters (Giordano 2005). When the supply fails to match myocardial demand cardiac contractile dysfunction occurs, and prolongation of this mismatch may lead to apoptosis and necrosis (Huang et al. 2004). Gene expression studies of mouse heart after chronic constant or intermittent hypoxia have also been explored in the past (Fan et al. 2005); however, effects of AHH on murine heart have been explored for the first time in this study.

Kidney

A remarkable paradox concerning the oxygen supply of the kidney is that the tissue oxygen tension within the kidney varies considerably. The partial pressure of oxygen is ~50 mmHg in the cortex while it ranges from 10-20 mmHg in the medulla. Medullary hypoxia results from the relatively low blood flow through the medulla. Also within the medulla, tubules and vasa recta are arranged in a hairpin pattern to maximize the concentration of urine by countercurrent exchange. Oxygen diffusion from descending to ascending vasa recta leaves the outer medulla deficient in oxygen. The high metabolic demands of medullary cells, particularly the medullary thick ascending limbs (mTAL) also accounts for the medullary hypoxia (Neuhofer and Beck 2006; Brezis et al. 1984; Brezis and Rosen 1995). The kidney thus operates under hypoxic conditions even under normal circumstances. Any stress such as acute hypoxia should affect the expression profile of genes to cope with hypoxia. When mammals are exposed to HA hypoxia, they exhibit certain physiological
responses, such as the production of EPO, a glycoprotein produced primarily by the kidney which plays the primary role in regulating red blood cell production (Krantz 1991; Jacobson et al. 1957).

Lungs

Although the percentage of oxygen in inspired air is constant at different altitudes, the fall in atmospheric pressure at higher altitude decreases the partial pressure of inspired oxygen and hence the driving pressure for gas exchange in the lungs. Venous blood doesn’t contain much oxygen, because it is returning from the body tissues, where oxygen has been used up. It is pumped through the lungs by the heart, and as it passes through the lungs it is exposed to fresh air in alveoli. When the blood passing through an area of lung isn’t picking up enough oxygen, the blood vessels carrying that blood tighten, so that less deoxygenated blood can get through the lungs. This is called hypoxic pulmonary vasoconstriction. The purpose of hypoxic pulmonary vasoconstriction is unclear. It may help match ventilation and perfusion within the lung, but in hypoxia of altitude the reflex leads to pulmonary hypertension and is associated with HAPE (Peacock 1998).

Brain

Hypoxia has progressive effects on the functioning of the central nervous system. Accidents that occur at extreme altitude on Everest and other mountains may be due to poor judgment as a consequence of hypoxic depression of cerebral function (Peacock 1998). The brain exhibits a remarkable capacity of structural and metabolic response to prolonged hypoxia. One of the most dramatic structural responses is the considerable remodeling of the cerebral microvascular network (Chavez et al. 2000).

1.6 OXIDATIVE STRESS AT HIGH ALTITUDE

ROS are oxygen molecules in different states of oxidation or reduction, as well as, compounds of oxygen with hydrogen and nitrogen. Oxygen is central to
the generation of ROS, several of which play vital roles in vascular physiology and pathophysiology; the most important of which are nitric oxide (NO’), superoxide (O2’•−), hydrogen peroxide (H2O2) and peroxynitrite (ONOO’•−) (Hool 2006; Droge 2002). ROS-linked injury is mainly due to hydroxyl radical (OH’•−), produced by the Fenton reaction i.e. H2O2 + Fe2+ → OH’− + OH’− + Fe3+ (Hippeli and Elstner 1999). Under aerobic conditions, most of free iron ions are in the oxidized form (Fe3+). However, under anaerobic conditions Fe2+ autoxidation ceases and Fe3+ is immediately reduced to Fe2+. The appearance of Fe2+ becomes dangerous as re-oxygenation activates the Fenton reaction and results in OH’•− formation (Skulachev 1997). At HA, less oxygen is available to terminally accept electrons from oxidative phosphorylation. Hypoxia partially inhibits mitochondrial electron transport, producing redox changes in the electron carriers and increase the generation of ROS at Mitochondrial Complex III (Chandel et al. 1998; Chandel et al. 2000).

HA is a multistress environment constituting, apart from hypoxia, several other stressors, such as extreme cold, ionizing radiations, physical exertion, and mental stress. Each of these stressors is capable of generating free radicals on its own (Vij et al. 2005). Some of the potential contributors to oxidative stress at altitude are shown in Table 1.1 (Askew 2002). Strengthening the antioxidant defense could be an effective strategy to prevent free-radical-mediated pathophysiological alterations and quicken acclimatization to oxidative stress (Vij et al. 2005).
### Table 1.1: Potential Contributors to Oxidative Stress at Altitude

<table>
<thead>
<tr>
<th>Stressor</th>
<th>Description</th>
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<tbody>
<tr>
<td>1. Exercise</td>
<td>Exercise is associated with free radical production from mitochondrial superoxide production</td>
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<tr>
<td>2. Ultra violet light</td>
<td>UVA and UVB can penetrate the epidermis and cause damage to underlying tissue</td>
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<tr>
<td>3. Lack of dietary antioxidants</td>
<td>More frequently than not, fresh fruits and vegetables are not an option at altitude, hence the diet may lack antioxidant nutrients</td>
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<tr>
<td>4. Catecholamines</td>
<td>Catecholamine production increases with hypoxia and work at altitude. Auto-oxidation of catecholamines can generate superoxide</td>
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<tr>
<td>5. Anoxia/reoxygenation</td>
<td>Similar to ischemia/reperfusion, flooding anoxic tissue with oxygen can lead to a burst of free radical production</td>
</tr>
<tr>
<td>6. Hypoxanthine &amp; xanthine oxidase</td>
<td>Hypoxia results in an accumulation of hypoxanthine (formed as a stepwise breakdown product of ATP) which is converted to xanthine and uric acid by xanthine oxidase when reoxygenation occurs, giving rise to superoxide</td>
</tr>
<tr>
<td>7. Reductive stress</td>
<td>Redox potential of cells is altered by hypoxia. Mitochondrial respiration is decreased leading to a build up of reducing equivalents that cannot be transferred to oxygen at cytochrome oxidase resulting in direct single electron reduction of molecular oxygen to form superoxide</td>
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1.7 ENDOPLASMIC RETICULUM STRESS

Any disturbance in the normal functioning of endoplasmic reticulum (ER) cause accumulation of unfolded proteins, triggering an evolutionarily conserved response, termed the unfolded protein response (UPR). The UPR aims to establish ER homeostasis by inducing the expression of genes that enhance the protein folding capacity of the ER, and promote ER-associated protein degradation to remove misfolded proteins. Excessive and prolonged ER stress may lead to apoptosis (Kaufman 2002; Schroder and Kaufman 2005; Xu et al. 2005). Glucose Regulated Protein 78 (Grp78), the most abundant ER chaperone, uses the energy from ATP hydrolysis to promote folding and prevent aggregation of proteins within the ER. When misfolded proteins accumulate, Grp78 releases critical transmembrane ER signaling proteins (Fig. 1.5) like PKR-like ER kinase (Perk), Inositol-requiring enzyme 1 (Ire1), and Activating transcription factor (Atf) 6, allowing their aggregation and launching the UPR (Xu et al. 2005; Schroder and Kaufman 2005; Kaufman 2002). Upon release from Grp78, Atf6 is transported to the golgi where it is cleaved by S1P/S2P proteases to yield the active transcription factor. Activated Atf6 directly modulate transcriptional induction of UPR target genes. The mRNA of X box protein 1 (Xbp1), a transcription factor, is induced by Atf6. Activated Ire1 splices Xbp1 pre-mRNA to remove a 26 bp intron to yield the Xbp1 mRNA which is translated to yield a more potent transcription activator. Xbp1 induces expression of genes involved in restoring protein folding or degrading unfolded proteins (Yoshida et al. 2001; Lee et al. 2003). Perk, a type I transmembrane kinase phosphorylates eukaryotic translation initiation factor 2α (eIF2α) resulting in inhibition of protein synthesis (Prostko et al. 1993; Harding et al. 1999). Hypoxia induced translational attenuation is also brought about by Perk via phosphorylation of eIF2α (Koumenis et al. 2002). Notably, an earlier study on several cell lines reported high phosphorylation of eIF2α after 1-2 hours exposure to hypoxia, with a gradual decline in the phosphorylation by the 8th hour (Koritzinsky et al. 2006). Perk represses translation of most mRNAs but selectively increase translation of Atf4. Atf4 regulates the promoters of genes involved in UPR and has been reported to be translationally upregulated under hypoxic conditions in a Perk-dependent manner (Harding et al. 2000; Blais et al. 2004).
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Fig. 1.5: Schematic Presentation of the Mammalian UPR. The three ER stress sensors Perk, Ire1 and Atf6 remain associated with Grp78. As unfolded proteins accumulate, Grp78 dissociates from the three sensors, thus freeing them for activation.

(Karar J et al., FEBS Lett. 2008;582:2521-26)
1.8 DIFFERENTIAL GENE EXPRESSION TO STUDY HYPOBARIC HYPOXIA

Mammalian cells require a constant supply of oxygen to maintain energy balance. Cells, tissues, and organisms are said to be hypoxic when they receive less than normal levels of oxygen. Given the central role of oxygen in the production of ATP through oxidative phosphorylation, it is critical for cells and tissues to respond rapidly to hypoxia. Sustained hypoxia can result in cell death (Seta and Millhorn 2004). It is therefore not surprising that sophisticated adaptive mechanisms have evolved that enhance cell survival during hypoxia. During the past few years, there have been a growing number of reports on hypoxia-induced transcription of specific genes that mediate such cellular functions as erythropoiesis, pulmonary ventilation and blood flow, angiogenesis, and energy metabolism (Ning et al. 2004; Ganfornina et al. 2005). The advent of microarrays provided the researchers an opportunity to study gene expression profiling of thousands of transcripts simultaneously. Microarrays have been used to study differential gene expression in rodent models of myocardial ischemia (Simkhovich et al. 2003; Lyn et al. 2000).

Microarray analyses have been increasingly used to identify hypoxia-responsive signal transduction pathways and genes that confer the hypoxia-tolerant phenotype (Seta and Millhorn 2004; Vengellur et al. 2005). It is obvious that a thorough understanding of how cells respond to hypobaric hypoxia will require an understanding of how different signaling pathways involved in the hypoxic response are coordinately regulated.

Classical gene expression analysis methods such as Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR) and nuclease protection assays, are best suited for analyzing a limited number of genes and samples at a time. Microarray analysis has emerged in the last few years as a flexible method for analyzing large numbers of nucleic acid fragments in parallel.

1.8.1 Microarray

All microarray systems share the following key components:

- the array, which contains immobilized nucleic acid sequences, or ‘targets’
- one or more labelled samples or ‘probes’, that are hybridized with the microarray
- a detection system that quantitates the hybridization signal
There are two extensively used formats: cDNA microarrays (Schena et al. 1995) and Affymetrix gene chips or oligonucleotide arrays (Lockhart et al. 1996). In the first format, many gene-specific polynucleotides derived from the 3’ end of RNA transcripts are individually arrayed on a single matrix. This matrix is then simultaneously probed with fluorescently tagged cDNA representations of total RNA pools from test and reference cells, allowing one to determine the relative amount of transcript present in the pool by the type of fluorescent signal generated. Relative message abundance is inherently based on a direct comparison between a ‘test’ cell state and a ‘reference’ cell state; an internal control is thus provided for each measurement. The scheme is similar when using radiolabelled probe, but it is not possible to carry out simultaneous hybridization of test and reference samples. In such cases, serial or parallel hybridization is required, introducing the possibility of higher variability in comparisons of expression level (Duggan et al. 1999).

Oligonucleotide arrays, on the other hand, employ a different system to label the complex probe. The arrays are designed based on sequence information alone and are synthesized in situ using a combination of photolithography and oligonucleotide chemistry (Lockhart et al. 1996).

**Array Fabrication**

Production of arrays begins with the selection of the ‘probes’ to be printed on the array. In many cases, these are chosen directly from databases including GenBank, dbEST and UniGene. Additionally, full-length cDNAs, collections of partially sequenced cDNAs (or ESTs), or randomly chosen cDNAs from any library of interest can be used. Arrays for higher eukaryotes are typically based on the EST portions of these projects, whereas for yeast and prokaryotes, probes are usually generated by amplifying genomic DNA with gene specific primers. cDNA arrays are produced by spotting purified PCR products (of approximately 0.6-2.4 kb) representing specific genes onto a matrix (Fig. 1.6). For both glass and membrane matrices, each array element is generated by the deposition of a few nanoliters of purified PCR product, typically of 100-500 mg/ml. Printing is carried out by a robot that spots a sample of each gene product onto a number of matrices in a serial operation. The types of membranes commonly used are nitrocellulose and charged nylon commercial varieties that are used for various blotting assays.
Fig. 1.6: Manufacture of cDNA Arrays and Affymetrix GeneChip Arrays
Glass-based arrays are most often made on microscope slides, which have low inherent fluorescence. These are coated with poly-lysine, amino silanes or amino-reactive silanes, which enhance both the hydrophobicity of the slide and the adherence of the deposited DNA. They also limit the spread of the spotted DNA droplet on the slide. In most cases, DNA is cross-linked to the matrix by ultraviolet irradiation. After fixation, residual amines on the slide surface are reacted with succinic anhydride to reduce the positive charge at the surface. As a final step, some percentage of the DNA deposited is rendered single-stranded by heat or alkali (Duggan et al. 1999).

The Affymetrix in situ process combines DNA synthesis chemistry with photolithography techniques from the microelectronic industry (Fig. 1.6). In this process, 50-terminal protecting groups are selectively removed from growing oligonucleotide chains in predefined regions of a glass substrate by controlled exposure of light through photolithographic masks. The glass substrate, or chip, is first covalently modified with a silane reagent to provide hydroxyalkyl groups, which serve as the initial synthesis sites. These sites are then extended with linker groups protected with special photolabile protecting groups. When specific regions of the surface are exposed to light (through masks), the protecting groups are selectively removed, allowing the sites to now be coupled with the next appropriate nucleoside phosphoramidite monomer. The monomers, which are also protected at their 50 position with a photolabile group, are coupled to the substrate using standard phosphoramidite DNA synthesis protocols. The cycles of photodeprotection and nucleotide addition are repeated to build any given array of oligonucleotide sequences. The direct, combinatorial synthesis of appropriate oligonucleotides based on sequence information alone eliminates the necessity of preparing and handling clones, PCR products and cDNAs. As the probes for each gene are specifically chosen the hybridization pattern and intensities can be interpreted in terms of gene identity and relative amount with no additional sequencing or characterization (Heller 2002; Lockhart et al. 1996).

**Target Labelling and Hybridization**

The targets for arrays are labelled representations of cellular mRNA pools. Typically, reverse transcription from an oligo-dT primer is used. This
has the virtue of producing a labelled product from the 3’ end of the gene, directly complementary to immobilized targets. Frequently, total RNA pools (rather than mRNA selected on oligo-dT) are labelled, to maximize the amount of message that can be obtained from a given amount of tissue. The purity of RNA is a critical factor in hybridization performance, particularly when using fluorescence, as cellular protein, lipid and carbohydrate can mediate significant non-specific binding of fluorescently labelled cDNAs to slide surfaces. Methods that produce multiple copies of mRNA using highly efficient phage RNA polymerases have been developed. A version of this approach, in which labelled target (cRNA) is made directly from a cDNA pool, having a T7 RNA polymerase promoter site at one end via in vitro transcription, has been applied to arrays. The cRNA is fragmented before hybridization (Duggan et al. 1999; Lockhart et al. 1996).

Image Analysis and Data Extraction

The highly regular arrangement of detector elements and crisply delineated signals that result from robotic printing and confocal imaging of fluor-detected arrays renders image data amenable to extraction by highly developed, digital image processing procedures. Grids specifying target locations can be readily overlaid on the images. Local sampling of background can be used to specify a threshold which true signal must exceed. In carrying out comparisons of expression data using measurements from a single array or multiple arrays, data is normalized using two strategies: based on a consideration of all of the genes in the sample, or on a subset expected to be unchanging over most circumstances (Duggan et al. 1999).

1.8.2 Gene Ontology

The result of a microarray experiment is a set of differentially regulated genes. A major challenge faced by the biologist is to translate this set of differentially regulated genes into a better biological understanding of the phenomenon that would allow a subsequent formulation of research hypotheses. Time and effort is invested in searching for all of the available information about each small area of research. This is hindered further by the wide variations in terminology that may be common usage at any given time, which inhibit effective
searching by both computers and people. The Gene Ontology (GO) project was
developed as a collaborative effort to address the need for consistent descriptions
of gene products in different databases and to provide a set of structured
vocabularys for specific biological domains that can be used to describe gene
products in any organism. This work includes building three extensive ontologies
to describe molecular function, biological process, and cellular component, and
providing a community database resource that supports the use of these ontologies

Onto-Express is a Java based program that makes use of the GO ontologies
or the gene associations provided by GO Consortium. This algorithm uses
UniGene accession or cluster identification numbers generated from microarray
investigations to search the public databases to return the functional profiles for
each genetic fingerprint (Khatri et al. 2002). Onto-Express starts by reading the
input file that exclusively contains single line entries of either accession or cluster
identification numbers. After creating the database, Onto-Express queries the
NCBI map viewer and retrieves the number of genes on each chromosome. If the
input file contains accession numbers, Onto-Express retrieves the cluster
identification number for each accession number and then builds a list of cluster
identification numbers. This eliminates redundancy, as multiple accession
numbers are often linked to a single cluster identification number. After building
the list of cluster identification numbers, Onto-Express retrieves the locus
identification number for each cluster. Onto-Express then queries the database
using each locus identification number to collect as much information as possible.
For each locus this includes biological process, biochemical function, molecular
function, cellular role, cellular component, and chromosomal location (Draghici et
al. 2003b; Khatri et al. 2002).

GenMAPP (Gene Microarray Pathway Profiler) is a program designed
for viewing and analyzing gene expression data in the context of biological
pathways (Dahlquist et al. 2002). GenMAPP extends the capabilities of the
known pathway resources like BioCarta, Kyoto Encyclopedia of Genes and
Genomes (KEGG) etc. by allowing users to modify pathways for their own
use, to design new pathways and to apply complex criteria for viewing gene
expression data on those pathways. GenMAPP represents biological pathways
in a special file format called 'MAPPs'. MAPPs are independent of the gene expression data and can be used to group genes by any organizing principle. Investigators can construct custom MAPPs with the graphics tools provided by the program, assigning each gene an identification (ID) from GenBank, SWISS-PROT or a user-defined ID system. To view gene expression data on MAPPs, the user imports the data in a comma-separated values format. GenMAPP has the flexibility to accept numeric and character data types, calculated values (such as p values), data from several experiments and data from both custom-spotted and commercial microarrays. GenMAPP displays gene expression data on pathways by color-coding the genes based on data and criteria provided by the investigator.

1.8.3 Real Time PCR

Real-time RT-PCR has been recognized as an accurate and sensitive method of quantifying mRNA transcripts. Real-time PCR collects data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity (Bustin 2000; Bustin 2002; Peters et al. 2004; Wong and Medrano 2005). The method allows the detection of amplicon accumulation since it is performed using fluorogenic probes or intercalating dyes such as SYBR Green. Intercalating dyes such as SYBR Green only fluoresce intensely when associated with double stranded DNA. Therefore, as double stranded PCR product accumulates, the level of fluorescence increases mirroring the template accumulation. The major drawback is that any double stranded product, including primer–dimers, will be detected and therefore false positives can occur. Applied Biosystems incorporates the internal passive reference dye ROX in all of its real-time PCR chemistries. It provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. The reference dye does not participate in the PCR reaction. This normalization corrects for fluorescence fluctuations that are caused by changes in reaction concentration or volume. Fluorescent probes offer a more specific way of detecting the accumulation of amplicon, since these probes use sequence specific oligonucleotides coupled to fluorescent dyes. There are several types of probe available, including hydrolysis probes (Taq-Man Probes), molecular beacons and
scorpions. Probes of all types are more expensive than reporter dyes but they allow sequence specific reporting, eliminating false positives due to non-specific product formation (Peters et al. 2004).

Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (Ct), the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct (Wong and Medrano 2005).

**Types of Real Time PCR**

Two types of quantitation are possible with the SYBR Green reagents (Wong and Medrano 2005):

**Absolute Quantification**

Absolute quantitation uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values.

**Relative Quantification**

During relative quantitation, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator. When using a calibrator, the results are expressed as a target/reference ratio. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays: standard curve method, comparative Ct ($2^{-\Delta \Delta Ct}$) method, Pfaffl method, Liu and Saint method etc.

The $2^{-\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression (Livak and Schmittgen 2001). The comparative Ct method is useful when a high number of targets and/or number of samples are tested. This method is very useful when using a high throughput strategy and when validating microarray results. The Comparative Ct Method uses arithmetic formulas to
achieve the result for relative quantitation. It is possible to eliminate the use of standard curves and to use the \( \Delta \Delta C_t \) method for relative quantitation as long as the PCR efficiencies between the target(s) and endogenous control(s) are relatively equivalent.

To determine if the two amplification reactions have the same PCR efficiency, one can look at how the \( \Delta C_t \) \( (C_t \) target \( - C_t \) reference) varies with template dilution. Assessing the relative efficiencies of the target amplification and the reference (endogenous control) amplification is achieved by running standard curves for each amplicon utilizing the same sample. The sample in the validation experiment must express both the target and reference genes. For example, a sample that ultimately is evaluated during experimentation (such as the calibrator) could be used. The \( C_t \) values generated from equivalent standard curve mass points (target vs. reference) are used in the \( \Delta C_t \) calculation. These \( \Delta C_t \) values are plotted vs. log input amount to create a semi-log regression line. The slope of the resulting semi-log regression line can be used as a general criterion for passing a validation experiment. In a validation experiment that passes, the absolute value of the slope of \( \Delta C_t \) vs. log input is < 0.1.

**Calculations of \( 2^{\Delta \Delta C_t} \) method**

1. The \( \Delta C_t \) value is calculated as \( \Delta C_t = C_t \) target \(- C_t \) reference
2. The standard deviation (s) of the \( \Delta C_t \) is calculated from the standard deviations of the target and reference values using the formula:
   \[ s = (s_1^2 + s_2^2)^{1/2} \]
   where \( s_1 \) and \( s_2 \) are standard deviations of target and reference.
3. The \( \Delta \Delta C_t \) is calculated as \( \Delta \Delta C_t = \Delta C_t \) test sample \(- \Delta C_t \) calibrator sample
4. The calculation of \( \Delta \Delta C_t \) involves subtraction of the \( \Delta C_t \) calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of the \( \Delta \Delta C_t \) value is the same as the standard deviation of the \( \Delta C_t \) value
5. Finally the fold difference is calculated using the formula \( 2^{\Delta \Delta C_t} \).