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High altitude hypoxia: An intricate interplay of oxygen responsive macroevents and micromolecules

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Abstract

Physiological responses to high altitude hypoxia are complex and involve a range of mechanisms some of which occur within minutes of oxygen deprivation while others reset a cascade of biosynthetic and physiological programs within the cellular milieu. The O_2 sensitive events occur at various organisational levels in the body: at the level of organism through an increase in alveolar ventilation involving interaction of chemoreceptors, the respiratory control centers in the medulla and the respiratory muscles and the lung/chest wall systems; at tissue level through the pulmonary vascular smooth muscle constriction and coronary and cerebral vessel vasodilation leading to optimized blood flow to tissues; at cellular level through release of neurotransmitters by the glomus cells of the carotid body, secretion of erythropoietin hormone by kidney and liver cells and release of vascular growth factors by parenchymal cells in many tissues; at molecular level there is expression/activation of an array of genes redirecting the metabolic and other cellular mechanisms to achieve enhanced cell survival under hypoxic environment. Transactivation of various oxygen responsive genes is regulated by the activation of various transcriptional factors which results in expression of genes in a highly coordinated manner. There is thus an intricate cascading interplay of biochemical pathways in response to hypoxia, which causes changes at the physiological and molecular levels. Added to this interplay is the possibility of genetic polymorphism and protein changes to adapt to environmental influences, which may allow a variability in the activity of the pathway. Our understanding of these interactions is growing and one may be close to the precise combination of genetic factors and protein factors that underlie the mechanism of what goes on under high altitude hypoxic stress and who will cope at high altitude. (Mol Cell Biochem **253**: 287–305, 2003)

Key words: high altitude, hypoxia, physiological response, cellular response, gene expression, transcription factors, HA malady

Introduction

The medical definition of high altitude (HA) is an elevation of 2700–5500 m above sea level (SL) while extreme altitudes have an elevation beyond 5500 m. Atmospheric pressure progressively decreases with increase in altitude. The atmospheric pressure at SL (760 mmHg) enables oxygen to easily pass through lung membranes into the blood. At an altitude of 5500 m, the atmospheric pressure is only 380 mmHg i.e. nearly half of its normal SL value. Proportion of oxygen (O_2) and nitrogen (N_2) in the air remaining the same, the partial pressure of oxygen of inspired air (PIO_2) at this altitude is 80 mmHg (i.e. 21% of 380 mmHg) and partial pressure of oxygen in alveoli (PAO_2) is even lower at 45 mmHg. 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.

Responses to hypoxia can be divided into four categories [1] according to the duration of hypoxia: (a) acute hypoxia, from seconds to an hour or two; (b) chronic hypoxia from hours to many years (i.e. sea level residents going to altitude, mountaineers; (c) lifelong hypoxia (altitude residents who were born and brought up at altitude; (d) species who have lived at altitude for generations. Acute responses have also been categorised as the ones occurring over a time scale of seconds to minutes and chronic with time course of hours to

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days [2]. Responses to acute and chronic hypoxia will be dealt with in the present paper with an aim to connect the macrolevel O_2 dependent physiological events with the microlevel molecular events.

HA hypoxic stress usually manifests with the inability to do normal physical activities. O_2 deprivation rapidly reduces the supply of ATP, which if prolonged, can cause tissue death. It is not uncommon to observe detrimental effect of hypoxia on reasoning skills and judgment during brief exposures to hypoxia which often persist for several days even after descent to SL. Initial acute response to HA hypoxia leads to development of inefficient physiological responses: rapid breathing, increased circulation and build up of muscle lactic acid. Heart pumps harder to supply more O_2 to the cells leading to elevated pulse rate and blood pressure. Later, when hypoxic exposure become chronic, a more efficient response develops as acclimatization takes place. More red blood cells and capillaries are produced for carrying more oxygen. The vascular network of muscles is also increased which enhances the transfer of gases. Such responses are designed to increase tissue O_2 supply.

Acute and chronic responses to hypoxic stress

Acute responses to HA hypoxia entail urgent adaptation while chronic responses to HA hypoxia entail long term adaptation. Acute or urgent and chronic or long-term adaptation are two major stages in the development of individual adaptation which are of general nature and occur in response to any kind of stress [3]. Acute adaptation is provided by preexisting mechanisms and involves activation of stress limiting systems viz., mobilization of energy and structural resources and their transportation to the functional system responsible for adaptation; rapid changes in activities of major lipid dependent membrane proteins, enzymes, receptors and ion transport channels; increased synthesis of second messengers; and activation of cell metabolism. These mechanisms are followed by the stage of gene activation, which enables the transition from acute to long-term or chronic adaptation. The process involves successive coordinated activation initially of early regulatory genes that code for protooncogenes and stress proteins followed by activation of late structural genes coding for structural proteins. Acute and long term adaptations are successive stages of the same process although both of them involve different mechanisms and have different biological functions. Activation of early genes and increased synthesis of protooncogenes and stress proteins constitute a nonspecific link in adaptation because they occur in adaptation to different factors. Subsequent adaptation to late structural genes is specific and activated genes provide adaptation to specific factors. In the case of adaptation to hypoxia, genes coding for proteins involved in oxygen transport, growth of coronary blood vessels and erythropoiesis are activated.

Physiological responses to HA hypoxia: The macroevents

Lack of O_2 at HA generally triggers physiological mechanisms which primarily aim to protect the important determinants of O_2 transport to tissue, particularly the respiratory and cardiovascular adjustments.

Hyperventilation

This is a critical response to hypoxic exposure, in which O_2 intake is increased through control of ventilation. At SL with normal ventilation, partial pressure of O_2 in alveoli (PAO_2) is ~ 100 mmHg. As alveolar PAO_2 falls with increasing altitude, the partial pressure of CO_2 in alveoli ($PACO_2$) also falls indicating progressive hyperventilation. Without hyperventilation, survival would be impossible at HA. Hyperventilation lessens the arterial hypoxemia at any given inspired PO_2. This is an essential adaptation to both acute and chronic hypoxia. Increase in ventilation depends on the activity of peripheral chemoreceptors, particularly those within the carotid body, which detect changes in arterial blood O_2 concentration and relay sensory information to the brainstem neurons that regulate breathing [4]. Hypoxic stimulation of the peripheral chemoreceptors begins within a few seconds (circulation time from lung to carotid chemoreceptor) of hypoxic exposure and intensifies over the initial hours and days of exposure to HA. The reason for this gradual increase over time at HL is an increased 'sensitization' of the carotid chemoreceptors so that the sensory input to the medulla from the chemoreceptor continues to increase for the same level of arterial hypoxemia. In addition, pH (which was initially very alkaline because of the lowered PCO_2) gradually returns toward normal due to renal excretion of sodium-, potassium- bicarbonate [5–7]. This loosens one of the (alkaline) 'brakes' on ventilation.

Oxyhemoglobin association/dissociation

Characteristics of the oxyhemoglobin (HbO_2) dissociation curve are important determinants for the ability of the body to 'tolerate' hypoxia of HA [8, 9]. At any altitude above 10000 ft, the arterial PO_2 falls into the steep portion of the oxyhemoglobin curve, below the safety range of the plateau region. As a result of this, the percent hemoglobin saturation (SaO_2) in the arterial blood also declines precipitously with

further increases in altitude. As a matter of fact, arterial blood O_2 partial pressure (PaO_2) can be decreased by 30–35 mmHg (to ~ 60 PO_2) before percent HbO_2 saturation falls below 90%. Furthermore, because of hyperventilation at HA, arterial CO_2 partial pressure ($PaCO_2$) is reduced, arterial pH is increased and the dissociation curve is shifted towards left. This enables maintenance of higher arterial O_2 % saturation as well as O_2 content at any given PO_2 in arterial blood which leaves the lung.

Polycythemia

Polycythemia (increase in the red blood cell concentration of the blood) is an important feature of acclimatization to HA [10–12]. The hypoxic stimulus to the kidney and liver cells releases erythropoietin hormone which, in turn, stimulates the bone marrow to produce red blood cells. In sojourners at HA, bone marrow stimulation begins within the first day of ascent, however, until the first 5–7 days of hypoxic exposure, increase in red cell production is not very apparent. Increased red cell production leads to increase in hemoglobin concentration which signifies increased oxygen carrying capacity of the blood. This indicates thereby that although the arterial PO_2 and HbO_2 saturation are reduced at HA, the oxygen content of the arterial blood is close to normal.

Blood flow

The normal hemodynamic response to acute hypoxia is an increase in heart rate (HR) [13–16]. This is due to redistribution of blood flow to organs which require a greater O_2 dependency e.g. skeletal muscles, kidneys, intestines and the brain. Increase in heart rate increases the cardiac output and helps restore the systemic O_2 transport. After a few days in hypoxia, cardiac stroke volume falls and cardiac output returns close to its sea-level values which may partly be due to a decreased plasma volume caused by increased diuresis at high altitude [17].

Hypoxia also selectively dilates some vascular beds in the brain and the coronary circulation. Increase in systemic and cerebral and coronary blood flow tends to counteract, in part, the reduction in oxygen transport imposed by reduced HbO_2 saturation. Brain blood flow is rigorously defended under conditions of both acute and chronic hypoxia through cerebral vasoconstrictor and vasodilator responses. The vasodilation of local hypoxia is counteracted by the vasoconstrictive effects of local hypocapnia, but the net effect is an increase in cerebral blood flow in hypoxic environments. This kind of attempt to protect cerebral O_2 transport in hypoxia is especially important because brain monoamine and neurotransmitter metabolism is especially sensitive to O_2 deficit. Chronic hypoxia has been shown to alter both these responses in isolated cerebral vessels [18]. Such effects may be important in individuals who develop disorders involving cerebral circulation under condition of acute or chronic hypoxia. Positron emission tomography (PET) for measurement of regional difference in cerebral blood flow showed an excessive increase in the hypothalamus during acute exposure to hypoxia [19].

Capillary to mitochondrial O_2 diffusion

Mitochondrial volume in human skeletal muscles decrease with HA acclimatization resulting in a decrease in absolute mitochondrial volume by nearly 30% [20]. There is an increase in capillary density (number of capillaries per unit area of distance) in skeletal muscle caused by reduction in diameter of muscle fibre [21, 22] which facilitates tissue diffusion of O_2. Myoglobin content increases both in skeletal and heart muscle which further facilitates O_2 diffusion from capillary to mitochondria and also acts as O_2 store in muscle for short periods of severe O_2 deprivation.

Regulation of substrate metabolism

Altitude and hypoxia alter regulation of substrate metabolism to favor carbohydrate (CHO) oxidation. In order to accommodate for the available supply of ATP and limitation of oxidative phosphorylation due to decrease in available energy, cells increase anaerobic glycolysis by upregulating glycolytic enzymes and decreasing the activity of some proteins that are ATP-consuming, e.g. Na^+-K^+ ATPase. Acclimatization to HA (4300 m) results in increased utilization of blood glucose [23] as well as greater dependence on blood glucose [24]. Recently it has been shown that acclimatization to HA selectively decreases key enzymes in fat utilization and oxidation in heart, liver and skeletal muscles [25]. Greater dependence on blood glucose rather than fatty acid metabolism probably assists in maintaining homeostasis by optimizing the energy yield per unit of oxygen. CHO oxidation would generate more ATP per molecule of O_2 consumed and can be metabolized non-oxidatively to yield ATP and lactate. However, some studies have provided indirect evidence to suggest that acclimatization to HA results in an enhanced rate of fat utilization, thereby sparing muscle glycogen stores [26].

Cellular and molecular responses to HA hypoxia

Initiation of the hypoxic responses encompassing the physiological manifestation at macro level at one end and micro-

molecular level at the other requires the existence of a fundamental cellular O_2 sensing mechanism which should be capable of detecting the fall in PO_2 and subsequently initiate an appropriate cascade of events that could culminate in the activation of particular functional response. Multicellular organisms are endowed with an elaborate mechanism for O_2 sensing and signal transduction [2]. Initially O_2 sensing was solely attributed to specialized chemoreceptor cells located in chemoreceptor organs (carotid, aortic or neuroepithelial cell bodies) [4] which can respond to decrement in O_2 tension in course of seconds or minutes with changes in their excitability, contractility or secretory activity. It is now, however, appreciated that all nucleated cells in the human body sense O_2 concentration and respond to reduced O_2 concentration both acute or chronic in duration.

In acute HA exposure in lowlanders, hypoxia defenses are proposed to be initiated by the several O_2-sensing, signal transduction pathways which can be described as five general hypoxia response systems: (i) O_2 sensors in the carotid body [27] initiate the hypoxic ventilatory response (HVR) [28] that helps to compensate for O_2 shortage despite risk of alkalosis [29] (ii) O_2 sensors in the pulmonary vasculature [30] initiate regulation of the hypoxic pulmonary vasoconstrictor response thus offering adjustments in lung perfusion [31, 32] (iii) O_2 sensors in the vasculature of other tissues activate the expression of vascular endothelial growth factor-1 [33] with its receptor [34] and thus initiate angiogenesis especially in the heart [35] and probably in the brain [36] (iv) O_2 sensors in kidney and liver activate expression of erythropoietin leading to the process of RBC mass upregulation [37] and (v) tissue-specific O_2-sensing and signal transduction pathways lead to metabolic reorganization [38] at least in part by altering expression rates of hypoxia-sensitive genes for metabolic enzymes and metabolite transporters [39].

Molecular basis of O_2 sensing

The identity of the mammalian oxygen sensor remains elusive. Many putative O_2 sensing mechanisms have been proposed [2, 4, 40, 41]. The O_2 sensor has been viewed as a hemoprotein that in the deoxy conformation activates the effectors either directly or through a signaling cascade. Existence of a heme-based O_2 sensor seems plausible because some heme proteins like hemoglobin bind O_2 with high affinity. A heme-based O_2 sensor may exist in erythropoietin-secreting cells [37]. Mitochondrial cytochrome particularly with a low affinity for oxygen can also serve as the oxygen sensor; although, identity of such a cytochrome is yet elusive. Spectral measurements of type I carotid body cells and hepatoma cell line Hep G2 cells provided evidence that the sensor is a cytosolic, membrane bound, multisubunit b-cytochrome that binds O_2 and reduces it to superoxide thereby generating reactive oxygen intermediate (ROI) [42]. Several non mitochondrial enzymes e.g. NADPH oxidase also contain heme and their enzymatic ability is critically dependant on the availability of O_2.

Two other potent systems which are proposed as O_2 sensors are the NADPH oxidase and the mitochondria [2, 43]. The NADPH oxidase is a multisubunit assembly consisting of a membrane-bound catalytic complex formed by $gp91^{phox}$ and $p22^{phox}$ subunits, a b_{558} cytochrome and several cytosolic regulatory subunits. The NADPH oxidase can transduce O_2 levels by changing the rate of production of superoxide anion (O_2^-) [44, 45]. Dismutation of O_2^- generates H_2O_2 which can oxidize transcription factors or ion channels. It has been proposed that NADPH oxidase produces ROS which in turn regulates membrane potential via K^+ channels [46]. However an immediate role of NADPH oxidase in O_2 sensing seems unlikely because cell lines deficient in gp91 and $p22^{phox}$ have been shown to display an unimpaired O_2-dependent modulation of gene expression [47]. Nevertheless, presence of a 'low output' NADPH oxidase isoenzyme as the O_2 sensor, which is closely related but clearly distinct from the high output oxidase as present in phagocytes, may not be ruled out [42]. NADPH oxidase isoforms with different $gp91^{phox}$ subunits as well as an unusual cytochrome aa3 with a heme a/a3 relationship of 9:91 have been suggested as putative O_2 sensor proteins influencing gene expression and ion channel conductivity [48]. Recent work on cardiomyocytes also point to the possible role of mitochondria as O_2 sensor wherein hypoxia is shown to result in a decrease in cytochrome oxidase V_{max} leading to accumulation of electrons in the reduced state and increased production of ROS [40, 49]. It is probable that an exponential increase of response with decreasing PO_2 is probably seen in cytosolic mitochondria and also in membrane bound NADPH oxidase. Nevertheless, identification of such an oxidase, either mitochondrial or cytosolic, which is critical for O_2-dependent gene expression via the PO_2-dependent production of ROS is still elusive. Image capture of formation of ROS by perinuclear Fenton reaction through 1 and 2 photon confocal microscopy has shown both mitochondrial and non mitochondrial generation of ROS [48]. Interestingly mitochondrial DNA-less *rho*(o) cells were shown to have a normal response to hypoxia [50] implying thereby that ROS may not be the only signaling molecule in the hypoxic signaling cascade.

Signal transduction in response to hypoxia

Primary function of signal transduction pathways is to activate protein factors in the nucleus that are involved in transcription which ultimately regulate the gene expression. During the

past few years there has been an enormous progress in understanding the molecular steps that link an initial signaling event with specific alteration in gene expression.

Growing body of evidences suggest that signal transduction by hypoxia involve both redox chemistry and protein phosphorylation; there are, however, gaps in our understanding about these mechanisms. The key event in O_2 signaling pathway has been proposed to be the generation of OH· from H_2O_2. that triggers gene expression [51, 52]. Role of H_2O_2 as a signaling molecule in O_2 response has been substantiated in studies investigating modulation by O_2 of aldolase A (ALDA), phosphoenolpyruvate kinase (PCK), glucokinase (GK) and tyrosine hydroxylase (TH) gene expression [53]. It is suggested that reactive oxygen intermediate (ROI) serves as a signaling molecule and a second messenger in O_2-dependant gene expression which provides a chemical link between alteration in intracellular O_2 concentration and responsive changes in the structure and function of appropriate transcription factors [39, 54].

Signal transduction in excitable cells is probably Ca^{2+} dependent. PC12 cells (*in vitro* model system for carotid body type I cells) have been extensively used for studying cellular and molecular events during hypoxic stress [55]. These cells depolarize during hypoxia due to inhibition of O_2-sensitive K^+ channels [56]. Depolarization activates voltage-dependant Ca^{2+} channels leading to translocation of Ca^{2+} from extracellular space, which in turn regulates gene expression via several known Ca^{2+} dependent pathways. Removal of Ca^{2+} from the extracellular milieu, inhibition of voltage-dependent Ca^{2+} channels, and chelation of intracellular Ca^{2+} prevents full activation of the tyrosine hydroxylase (TH) gene expression during hypoxia [57]. These findings suggest that membrane depolarization and regulation of intracellular free Ca^{2+} are critical signal transduction events that regulate the expression of late response tyrosine hydroxylase (TH) gene during hypoxia [58]. The *cis* elements of the TH gene contain sites for binding of transcription factors activator protein-1 (AP-1) as well as hypoxia-inducible factor-2 (HIF-2). PC12 cells, which have been used as a TH expression model system do not have HIF-1α. It is probable that during hypoxia, an increase in intracellular Ca^{2+} activates a signaling pathway that involves AP-1 dependent transcription. Activation of AP-1 in hypoxia requires prior induction of the immediate early genes *c-fos* and *JunB* which depend on the influx of extracellular Ca^{2+}. Intracellular pathways that link hypoxia to activation of c-*fos* gene expression involve L-type voltage-gated Ca^{2+} channel and is dependent on the activation of Ca^{2+}/calmoudolin-dependent protein kinases (CaMK). Both Ca^{2+}/cAMP-responsive (Ca/CRE) and serum-responsive (SRE) *cis* elements are found to be essential for transcriptional activation of c-*fos* by low O_2 [59].

Hypoxia-regulated protein kinases

Intracellular transduction mechanisms entail cascades of protein phosphorylation and dephosphorylation involving the interplay of a broad repertoire of kinases and phosphatases. The major signal transduction pathways that are activated by hypoxia in excitable cells are Ca^{2+}/calmodulin-dependent protein kinases (CaMK), Ca^{2+}/calmodulin dependent phosphatases and various isoforms of protein kinase C (PKC) [55]. PKC system appears to be a critical element in cellular adaptation to hypoxia. p42/p44 MAPK protein kinases are also activated by hypoxia which are critical for the hypoxia-induced transactivation of endothelial PAS-domain protein 1 (EPAS1)/HIF-2α, another hypoxia-inducible transcription factor [60]. Hypoxia, like various other stressors, also activates the p38 (p38α, p38β, p38δ, p38γ) signaling pathway, which is a part of the mitogen activated protein kinase (MAPK) superfamily. Two p38 isoforms, p38α and p38γ, are shown to be activated by hypoxia in the PC12 cell line [61]. Upstream signaling cascades that regulate p38 function have only been partially characterised. It has been shown that p38 kinases are phosphorylated and activated by upstream MAP kinase kinases (MKKs) including MKK3, MKK6 and possibly MKK4 [62]. Further upstream of the MKK lie a number of signaling molecules which include the Rho family of GTPases as well as the cell surface receptors which can potentially activate p38. p38γ is also regulated by the dopamine receptor and calcium following hypoxic exposure [63].

Both mitogen- and stress-activated protein kinases (MAPKs and SAPKs) have been identified as hypoxia-regulated protein kinases which regulate gene expression and cell function [64]. Another cytosolic serine/threonine kinase required for growth factor mediated cell survival, *Akt* (also termed protein kinase B or PKB) is activated by hypoxia. There are now increasing evidences that *Akt* and glycogen synthase kinase-3 (GSK-3) signaling pathways play a key role in cell adaptation and survival under hypoxic conditions [65]. Function of HIF-1 probably also depends on it being phosphorylated [66–68] although the precise signaling pathway as well as the link between sensor function and HIF-1α stability has not yet been well elucidated. A Rho family small GTPase Rac I has been proposed as a potential intermediate in the hypoxia signal transduction pathway [69]. Some reports have described that hypoxic induction of human vascular endothelial growth factor (VEGF) gene is through activation of tyrosine kinase *src* expression which is responsible for downstream phosphorylation events presumably through activation of HIF-1 [70]. Recent work of Jung *et al*. [71] demonstrate the role of tyrosine kinase cascade in hypoxic regulation of HIF-1α which is mediated via *Shc* adaptor protein in concert with *Ras* and *Raf*-1 in endothelial cell.

Under conditions of hypoxia, low blood flow or when glucose is limiting for cellular metabolism, a 5′-AMP activated protein kinase alpha 1 catalytic subunit (PRKAA1) belonging to serine-threonine family of protein kinases [72, 73] appears to act as metabolic stress-sensitive protein kinase which switches off biosynthetic pathways in response to fuel limitation and/or hypoxia. Decreased intracellular concentration of ATP in response to hypoxia has also been shown to regulate the mammalian target of rapamycin (mTOR) signaling [74] which plays a key role in hypoxia-triggered smooth muscle and endothelial proliferation and angiogenesis *in vitro* [75].

HA Hypoxia induced gene expression

Physiological manifestation of HA hypoxic stress would be regulated at the molecular level by the expression of specific target genes appropriate to the response. In the carotid body, hypoxia elicits rapid inhibition of conductance through potassium channel and induces expression of tyrosine hydroxylase (TH) gene which codes for the rate-limiting enzyme in the synthesis of neurotransmitter dopamine [76, 77]. Induction of tyrosine hydroxylase facilitates the control of ventilation via the carotid body. The hypoxic ventilatory response (HVR) is modulated by the dopamine D(2)-receptors (D(2)-R) in both the carotid body arterial chemoreceptors and the nucleus tractus solitarius (NTS). Chronic hypoxia has been shown to alter D(2)-R gene expression to initiate changes in D(2)-R modulation of HVR and enhance ventilatory acclimatization to hypoxia [78]. Expression of endothelin-1 gene is also induced by hypoxia in the carotid body which augments chemoreceptor responses by enhancing intracellular Ca^{2+} levels and stimulating proliferation of glomus cells [79, 80].

Alteration in oxygen tension up regulates the expression of genes involved in erythropoiesis, angiogenesis, tissue remodeling and vasomotor control. These include erythropoietin (EPO), transferrin, transferrin receptor, vascular endothelial growth factor (VEGF), VEGF receptor *flt-1*, basic fibroblast growth factor (bFGF), platelet derived growth factor A and B, insulin-like-growth factor 2 (IGF-2), endothelin-1 (EDN-1), nitric oxide synthase (NOS) and heme oxygenase-1 (HO-1) [2, 81]. Genes encoding enzymes responsible for glucose metabolism like glucose transporters GLUT-1 and GLUT-3, glycolytic enzymes aldolase A and C, hexokinase 1 and 2, glyceraldehydes 3-phosphate dehydrogenase, lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase 1, pyruvate kinase M, triphosphate isomerase are also induced by hypoxia [2, 81]. Regulation of number of genes which are expressed in endothelial cells are affected by tissue oxygenation which include platelet derived growth factor [82], interleukin-1α [83], interleukin-8 [84], endothelin-1, IGF binding protein 1-3, p21, p35srj etc. [81].

Transcription factors in HA hypoxia

Gene expression is regulated by the activation of suitable transcription factors which are proteins that bind to DNA near the transcription start site and bring about transactivation of specific responsive genes through mechanisms of DNA binding and subsequent assembly (dimer and/or tetramer formation). Structural features of transcription factors include sequence specific DNA binding domains (helix-turn-helix, zinc finger, B-Zip), activation domains (acidic domain, glutamine-rich domain, proline-rich domain), nuclear localization domain and dimerization domain. A wide range of transcription factors like HIF-1, HIF-2, AP-1, early growth response-1 (Egr-1), high mobility group (HMG) 1(y), nuclear factor interleukin-6 (NF-IL6) and nuclear factor kappa B (NFκB) etc. are suggested to be oxygen regulated [52, 113] although the mechanism by which hypoxia is sensed and signal is transduced remain enigmatic. Furthermore, the specific signaling pathways by which these various transcription factors control gene expression are also poorly understood.

Hypoxia inducible factor-1 (HIF-1)

HIF-1 was the first transcription factor which was shown to be essential for hypoxia induced transcription of the erythropoietin (EPO) gene in Hep3b cells [85]. It is likely that cells from a wide variety of tissues share a common mechanism of O_2 sensing and signal transduction leading to the activation of the transcription factor HIF-1 [86]. A number of target genes are activated by HIF-1 [81, 86].

HIF-1 is a heterodimeric protein composed of an alpha and a beta (also known as aryl hydrocarbon nuclear translocator (ARNT)) subunit both of which are members of the basic helix-loop-helix PER-ARNT-SIM (PAS)(bHLH/PAS) family of proteins [81]. HIF-1α and ARNT (HIF-1β) mRNA are expressed in most human and rodent cells. Heterodimer of HIF-1α and HIF-1β bind to DNA at sites represented by the consensus sequence 5′-RCGTG-3′ [87]. The HIF-1 binding site is present within a hypoxia response element (HRE), a cis-acting transcriptional regulatory sequence which can be located within 5′-flanking, 3′-flanking or intervening sequences of target oxygen responsive genes. The TAD-C at the COOH-terminal portion of HIF-1 binds specifically to a general transcriptional activator, p300, that participates in a number of biological functions such as induction of various tissue-specific enhancers, regulation of cell cycle, and stimulation of differentiation pathway. p300 is a very large protein

and closely homologous to adenosine 3′,5 cyclic monophosphate response element binding protein (CREB-1). p300 and human CREB-binding protein (CBP) interact with the HIF-1a in the hypoxia signaling pathway [88, 89]. p300 is thought to play a critical role in transducing the signal from HIF-1 enhancer complex to the apparatus responsible for the initiation of transcription. HIF-1α conditionally interacts via two distinct transactivation domains with the coactivators CBP1/p300, SRC-1 or transcription intermediary factor 2 (TIF2) in addition to the nuclear redox regulatory protein Ref-1, and these interactions are important for complete transcriptional activation of HIF-1α [90, 91]. It is possible that Ref-1 functions in hypoxic cells by targeting critical steps in the recruitment of the CBP-SRC-1 coactivator complex [91].

Isoforms of HIF-1α are termed HIF-2α (also called EPAS-1) and HIF-3α which have significant sequence homology with HIF-1α [92]. It appears that all three factors are regulated by hypoxia using similar mechanism. Regulation of HIF-1α expression and activity occurs at multiple level *in vivo*, including mRNA expression, protein expression, nuclear localization and transactivation. The α subunit remains stabilized under hypoxic conditions which is readily available for dimerization with the β subunit to activate transcription of hypoxia-responsive genes. The beta subunit is constitutively expressed under both normoxic and hypoxic conditions. Under normoxic conditions, the alpha subunit is rapidly ubiquitinated and degraded by the proteosome [93–95] which also requires the E3 ubiquitin ligase containing the von Hipple-Lindau (VHL) tumor suppressor protein (pVHL) [86]. Human pVHL binds to a short HIF-derived peptide when a conserved proline residue at the core of this residue is hydroxylated. Proline hydroxylation requires molecular O_2, Fe^{2+} as well a citric acid cycle intermediate 2-oxoglutarate and thus this protein modification may play a key role in mammalian O_2 sensing [96, 97].

Activator protein-1(AP-1)

Many of the late response genes like tyrosine hydroxylase that are activated during hypoxia depend on the activator protein-1 (AP-1) transcription complex and are shown to contain AP-1 consensus binding sites in their promoter region. The AP-1 transcription factor was originally described as a heterodimer of c-Jun and c-Fos [98, 99]. These basic region leucine-zipper (B-ZIP) transcription factors dimerize via their leucine-zipper domains, which in turn bring together their basic domains to bind DNA in a sequence-specific manner. In recent years several homologues of c-Jun (JunB and JunD) and c-Fos (FosB, Fra1 and Fra2) have also been shown to form heterodimers. In addition, members of the activating transcription factor (ATF) family, such as ATF2, ATF3 and ATF4, can also interact with members of the Fos and Jun family of proteins [100, 101]. The Jun and ATF proteins form stable homodimers as well as heterodimers, whereas c-Fos does not dimerize and is incapable of binding DNA as a homodimer [102, 103]. The current view is that AP-1 actually consists of several distinct homodimers or heterodimers composed of various members of the Fos, Jun and ATF B-ZIP subfamilies [104].

Genes that encode for c-*fos* and *jun*B transcription factor proteins are regulated by reduced O_2 tension [57]. The critical redox-sensitive site is a single conserved cystein residue in the DNA binding domain of Fos and Jun [105]. AP-1 heterodimers bind to DNA on a serum response element with the 5′-TGA(C/G)TCA-3′ sequence which is the consensus binding site for AP-1. Phosphorylation of AP-1 family members by kinases is required for transactivation activity.

Nuclear factor-IL6 (NF-IL6)

Nuclear factor-IL6 (NF-IL6), also known as CCAAT-enhancer-binding protein-β (C/EBPβ), is a basic-leucine-zipper transcription factor and activates interleukin-6 (IL-6*)* gene transcription in hypoxic pulmonary vascular endothelial cells (ECs) [106]. NF-IL6 binds to an IL-1 response element in the IL-6 gene apart from binding to regulatory regions of several acute phase and cytokine genes. The consensus recognition site is 5′-T(T/G)NNGNAA(T/G)-3′. It binds DNA as a dimer and can form stable heterodimers with C/EBPα, δ and γ. Expression of NF-IL6 is tissue specific and is expressed in low abundance in lungs, kidney and spleen. Stimulation of protein kinase C increases phosphorylation of NF-IL6 and enhances its transcriptional efficiency. NF-IL-6 mediated IL-6 gene product promotes the expression of adherence molecules that recruit activated leukocytes to the vessel wall, leading to vascular leakage and/or thrombus formation [107] thus protecting the integrity of the pulmonary vasculature under conditions of hypoxia or ischemia.

Early growth factor-1 (Egr-1)

Egr-1 (also known as ZIF268) is a zinc-finger transcription factor which is expressed in the lungs after exposure to acute hypoxia [108]. Upregulation of Egr-1 is found in lung smooth muscle cells as well as in mononuclear phagocytes. Egr-1 regulates the expression of downstream target gene, tissue factor gene, wherein Egr-1 interacts with its cognate DNA binding sites in the serum response region of the promoter of tissue factor gene [109]. Egr-1 mediated production of tissue factor (a procoagulant regulator) by monocytes leads to hypoxia-induced fibrin deposition in the pulmonary vasculature [110, 111]. Egr-1 and tissue factor expression have been shown to be co-induced in bronchial and vascular smooth

muscle and alveolar macrophages and marked vascular deposition of fibrin is observed in the lungs of wild-type mice subjected to hypoxia whereas none of these responses occur in knockout mice lacking expression of the gene encoding protein kinase C-β [112]. The mechanism by which hypoxia activates protein kinase C-β in mononuclear phagocytes is unknown but this activity seems to be crucial for the induction of Egr-1 and tissue factor production. Hypoxia-induced Egr-1 activity thus promotes pulmonary vascular thrombosis in contrast to action of NF-IL6 [113]. Egr-1 also mediates transcriptional activation of IGF-II gene in response to hypoxia [114].

Nuclear factor kappa B (NFκB)

Nuclear factor kappa B (NFκB) is a dimeric protein which is maintained in an inactive form in cytoplasm in most cells. It is activated by many different stimuli and activation is achieved by removal of an inhibitory protein complexed with it termed IκB. Upon cellular stimulation, IκB is phosphorylated and degraded resulting in rapid translocation of NFκB to the nucleus where it stimulates the expression of a wide variety of genes that particularly mediate cell's response to stress. Genes regulated by NFκB include cytokines, cytokine receptors, adhesion molecules, stress response genes, early response genes, cell surface receptors, growth factors and their modulators, other transcription factors etc. (Gilmore TD, http://people.bu.edu/gilmore/nf-kb/index.htm).

NFκB proteins are divided into two classes based on sequences C-terminal to Rel homology (RH) domain. Members of one class (p105, p100) have long C-terminal and multiple copies of ankyrin repeats which act to inhibit these molecules. Through limited proteolysis or arrested translation, members of this class become active, shorter DNA-binding proteins (p105 to p50, p100 to p52). The second class includes members C-Rel, RelB, RelA (p65) which contain C-terminal transcription activation domain. Members of first class form dimers with members of second class. p50-RelA (p65) heterodimer is one of the most avidly forming dimers and is the major NFκB complex in most cells (Gilmore TD, http://people.bu.edu/gilmore/nf-kb/index.htm).

A few physiologically relevant target genes of HA hypoxia

Erythropoietin

Erythropoietin (30.4 kDa glycoprotein hormone), which is a product of erythropoeitin (EPO) gene, is a key blood protein that regulates erythropoiesis and mediates some of the responses to altitude hypoxia [115–117]. Hypoxic regulation of EPO gene has been extensively studied. The human EPO gene spans an area of 3.6 Kbp in the genome and comprises five exons and four introns. Only a single copy of the human EPO gene has been mapped to chromosome 7pter-q2210 (www. ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). Extensive homology exists among the EPO genes of various species. Unlike most genes, the EPO promoter lacks the canonical TATA or CAAT sequences [39]. It contains three candidate nuclear hormone response half sites which do not contribute to hypoxic induction but are required for cooperation with the enhancer. Other elements in the EPO promoter include CACCC and GATA sites and a ribonucleoprotein binding site. A kidney-inducible sequence (KIE) is located between 9.5 and 14 kb upstream of the human EPO gene and a negative regulatory element (NRE) is found 0.4–6 kbp upstream. The *cis*-element most critical to the hypoxic induction of the EPO gene is a conserved 40-bp element in the 3′-flank, 120 bp dowstream of the polyadenylation site. This element confers a 4–40 fold induction in response to hypoxia [39].

The EPO 3′-enhancer is composed of three interacting parts: a highly conserved 5′ 13-mer sequence which binds to HIF-1; a middle portion consisting of three CA repeats which is necessary for hypoxic induction and a highly conserved 3′-portion which is a double direct repeat of a consensus steroid hormone response elements (HREs), separated by a 2-bp spacer just downstream of HIF-1 binding site [39, 118]. Specific nuclear receptors bind to these half sites and have a marked impact on hypoxic induction and tissue specificity [119]. The immediate early response genes like c-fos, c-jun, c-myc, EGR-1 are probably not involved in the sequence of events by which hypoxia stimulates Epo gene expression [120].

The regulation of EPO gene expression is not yet fully understood. Under conditions of hypoxia the expression of EPO gene is activated via binding of hypoxia-inducing factor-1 to the 5′-end of the hypoxia-inducible enhancer located at the 3′-flanking region of the EPO gene. Additional transcriptional factor, such as hepatic nuclear factor-4 (HNF-4) bind constitutively to the tandem repeat hormone response elements (HRE), which acts synergistically with the enhancer to promote transcription of the EPO gene under conditions of hypoxia [118]. Synthesis of erythropoietin is regulated at both transcriptional and post-transcriptional levels. An EPO-RNA binding protein (ERBP) which specifically binds to the 3'-untranslated region of EPO mRNA is likely to be involved in the regulation of EPO mRNA stability.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis [121]. Encoded in a single gene,

the human VEGF gene is organized in eight exons, separated by seven introns, with its coding region spanning approximately 14 kb. The human VEGF gene has been assigned to chromosome 6p21.3 (www. ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). Analysis of the VEGF gene promoter region reveals a single major transcription start site that lies near a cluster of potential SP-1 factor-binding sites. VEGF promoter lacks the canonical TATA or CAAT boxes [40] but several potential binding sites for the transcription factors AP-1 and AP-2 are present in the promoter region [122] which may or may not be important for hypoxic induction. It also contains conserved AP-2 site and a cluster of conserved SP-1 sites whose functions are not yet known. A number of consensus HIF-1 binding sites are present in the 5′- flanks of the VEGF gene. One of these sites, the hypoxia responsive enhancer (28 bp) is conserved which is present 900 bp upstream of the transcriptional start site in the rat genome [123]. Just like the EPO enhancer, this site is only a few base pairs 5′ of an imperfectly conserved CACAG sequence. A conserved AP-1 binding site is present in the promoter which contributes to hypoxic induction because expression of Fos and Jun is increased in many cells which are subjected to hypoxia. However, hypoxic induction of VEGF may also be independent of AP-1 regulation [124]. VEGF and EPO synthesis share several regulatory mechanisms [125] including the participation of a hemoprotein as the O_2 sensor and *cis*-acting elements that bind to HIF-1.

cDNA sequence analysis of a variety of human VEGF clones indicate that VEGF mRNA may exist as one of four different splice variants ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) [126] with the 165-amino acid isoform ($VEGF_{165}$) being the predominant secreted form. It is now well established that alternative exon splicing of a single VEGF gene is the basis for this molecular heterogeneity. VEGF mRNA is regulated posttranscriptionally and stability of the mRNA is enhanced by hypoxia. The 3′ UTR (untranslated region) of VEGF mRNA contain multiple AUUUa and polypyrimidine sequence motifs [127]. A 40 bp functional HuR binding site is present in VEGF mRNA 3′-untranslated region which seems to mediate stabilization of the VEGF [128].

Tyrosine hydroxylase

Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine (dopa) the first and rate-limiting step in the biosynthesis of catecholamine neurotransmitters. The human tyrosine hydroxylase (TH) gene is located on chromosome 11p15.5 and comprises 2838 bp (www. ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). It is split into 14 exons and 13 introns. Analysis of reporter-gene constructs reveal that increased transcription of the TH gene during hypoxia is regulated by a region of the proximal promoter that extends from –284 to –150 bases, relative to the transcription start site [129]. This region of the gene contains a number of cis-acting regulatory elements including AP1, AP2 and hypoxia-inducible factor-2 (HIF-2). Hypoxia-induced binding occurs at both the AP-1 and HIF-2 sites. AP-1 site is required for increased transcription of the TH gene during hypoxia. A common protein has been shown to bind to the mRNAs encoding EPO, VEGF and TH [130]. Through alternative splicing from a single gene, four types of human TH mRNA are produced.

Endothelin 1

Product of endothelin 1 gene (EDN-1) is a 21-residue peptide which is synthesized and secreted by vascular endothelial cells. This is the most potent endogenously produced vasoconstrictor which acts through multiple systems: systemic, pulmonary and coronary vasculature [131]. Hypoxia induces expression of both EDN-1 and platelet derived growth factor-β (PDGF-β) chain gene in vascular endothelial cell [82, 132]. Circulating EDN-1 levels which are increased under hypoxia are of pulmonary origin [133]. Combined actions of EDN-1 and nitric oxide are probably important factors in regulating vascular tone and blood pressure.

EDN-1 gene (chromosomal location 6p24.1) promoter contains HIF-1-binding site which is required for hypoxia-induced transcription [134]. Apart from the HIF-1 binding site, an additional 50 bp of flanking sequences that include binding sites for the factors AP-1, GATA-2, and CAAT-binding factor (NF-1) are also present. Mutation of any one of these sites or the HIF-1 site eliminates induction by hypoxia. Mutations of the AP-1 and GATA-2 sites, but not the HIF-1 site, are complemented by overexpressing AP-1, GATA-2, HIF-1α, or the activator protein p300/CBP, restoring the response to hypoxia. Binding studies *in vitro* have confirmed physical associations among GATA-2, AP-1, and HIF-1 factors. Regulation of the EDN-1 promoter by hypoxia in non-endothelial cells requires overexpression of GATA-2 and HIF-1α. The results support essential roles for AP-1, GATA-2, and NF-1 in stabilizing the binding of HIF-1 and promoting recruitment of p300/CBP to the ET-1 hypoxia response complex [135].

Genes involved in glucose metabolism

Most mammalian cells transport glucose through a family of membrane proteins known as glucose transporters (Glut or SLC2A family). Molecular cloning of these glucose transporters has identified a family of closely related genes (GLUT-1 to GLUT-14), chromosomal location 1p35-p31.3 (2865 bp) that encode at least 9 proteins. GLUT-1 and GLUT-3 are induced by hypoxia, whereas expression of GLUT-2 is de-

creased. GLUT-1 mediates glucose transport into red cells and throughout the blood brain barrier. It is ubiquitously expressed and transports glucose in most cells. GLUT-2 provides glucose to the liver and pancreatic cells while GLUT-3 is the main transporter in neurons. GLUT-1 expression is dually controlled via HIF-1 and reduced oxidative phosphorylation [136]. Regulation of GLUT-1 depends on a complex array of *cis*-acting elements and transcription factors [136, 137]. In addition to transcriptional regulation, hypoxia also increases the steady state GLUT-1 mRNA levels through increased mRNA stability similar to other HIF-1 responsive genes [138].

Regulation of genes encoding glycolytic enzymes is complex and not well understood. Isoenzyme-specific regulation by hypoxia is observed for genes encoding phosphofructokinase, aldolase and lactate dehydrogenase [139]. Analysis of *cis*-elements of these glycolytic enzymes show a common mode of hypoxic induction. The HIF-1 binding consensus sequence is present in the 5′-flank of the human phosphoglycerate kinase 1, mouse lactate dehydrogenase A and human enolase 1 gene and in the intervening sequences of mouse phosphofructokinase L and human aldolase A genes [140, 141].

Expression of phosphoenolpyruvate carboxykinase (PKC) is downregulated in response to hypoxia [142] thereby favouring gluconeogeneis. The promoter of PKC gene has two glucocorticoid receptor sites. Transcriptional activation of PKC by glucocorticoids depend on the binding by two orphan receptors, HNF-4 and COUP which are also shown to play a role in EPO gene expression.

Nitric oxide synthase

Three isoforms of nitric oxide synthase (NOS) gene have been characterized which include neuronal NOS or NOS I, inducible NOS or NOS II and endothelial NOS or NOS III [143]. NOS I and NOS III are constitutively expressed [144] and are responsible for basal generation of NO which is critical for basal blood flow and pressure regulation across many organs and species. NOS II is inducible wherein the isozyme is generally not present in the cell and production of NO by inducible NOS is enhanced by increasing the amount of NOS protein through augmented transcription and translation. Stress induction of genes coding NOS occur as a result of activation of the transcription factor NFκB. NOS inhibitors augment the respiratory responses to hypoxia, an effect that may be due to blockade of NOS in the carotid body and central neural structures that regulate breathing during hypoxia [145, 146].

Adaptation to hypoxia increases both the expression of NOS gene [147] as well as the NOS content [148]. NOS expression is induced in the endothelium of pulmonary resistance vessels, in the smooth muscle of large and small pulmonary vessels and in bronchial smooth muscles [148]. All three isoforms of NOS are present in the lung and have been reported to increase in chronic hypoxia-induced models of pulmonary hypertension [148, 149]. Increase in NOS expression occurs as early as 24 h after hypoxia and the increased NOS expression continues in a manner that precedes and progresses with the development of pulmonary vascular remodeling [150]. Stress induced increase in NO synthesis has been shown to occur due to the activation of both preexisting NOS and NOS formation *de novo* [3]. It has been hypothesized that in conditions of hypoxia, NO formation may not entirely be dependant on NOS and can be formed from nitrates by nitrite reductase reactions.

The NOS genes are complex and each of the three classes of NOS represent separate gene products [143]. Human NOS I gene (chromosomal location 12q24.2-24.3) having 28 exons and 27 introns is spread over 100 kb. The mRNA for NOS I codes for 1429 amino acids (aa) with the resultant monomeric protein of 160 kDa. NOS II gene (chromosomal location 17q11.2) has 26 exons and 25 introns and is spread over 37 kb. It codes for 1154 aa resulting in a protein of approximately 135 kDa. NOS III gene has 26 exons and 25 introns over 21 kb; the mRNA codes for 1205 aa with the protein molecular weight of approximately 135 kDa. Various genetic polymorphisms have been noted in the DNA sequence which can create different mRNAs for the same gene [151]. In addition, the native RNAs transcribed from these genes combine in different ways to form mature mRNA. However it is unclear if the differences in mRNA have any impact on the enzymatic activity.

The NOS I isoform is expressed in several neuronal structures associated with respiration e.g. the sensory nerve fibres innervating the carotid body, the primary sensory organ that monitors arterial oxygen [152, 153] as well as in the neurons in the nucleus tructus solicitus (NTS), the region of the brain stem that receives and integrates afferent inputs from the peripheral chemoreceptors [154, 155]. Prabhakar *et al*. [156] demonstrated that up-regulation of NOS I leads to increased generation of nitric oxide, which in turn may contribute to the readjustments of cardio-respiratory systems during the early stages of chronic hypoxia. NOS I mutant mice have been shown to exhibit augmented respiratory responses to hypoxia [157]. It has been observed that 12–24 h of hypobaric hypoxia selectively activates NOS I gene expression, which is reflected in an increase in NOS I protein in central and peripheral neurons. Enhanced NOS I expression is often associated with coinduction of transcription factors c-jun and c-fos [144].

NOS III is primarily localized to the endothelium of many blood vessels, including the vasculature supplying the carotid body and cerebral blood vessels [158]. NO generated from NOS III regulates the blood flow through control of vascu-

lar tone [159, 160]. Decrease in vasomotor tone, due to decrease in O_2 supply, results in an increase in cerebral blood flow (CBF) to match with O_2 requirement of the brain [161, 162]. Mechanism underlying hypoxia induced vasodilation in selective vascular beds is not understood fully. Phase contrast magnetic resonance imaging shows that hypoxia-induced cerebral vasodilation in human is mediated by nitric oxide [163]. Wang *et al*. [164] suggested that variation in the endothelial NO synthase (NOS 3) gene sequences might affect NO production. NOS III mutant mice have also been shown to exhibit reduced respiratory responses to hypoxia probably through blunted carotid body sensitivity [165].

In a chronic hypoxic rat model, hypoxia increased NOS II mRNA and protein levels 1.9 and 1.4 fold respectively [149]. One mechanism by which hypoxia may increase NOS II gene expression is through induction of HIF-1 [166]. The 5′-flanking region of the murine NOS II gene is shown to contain a DNA sequence that is functionally essential for hypoxia-induced transcriptional activation in pulmonary artery endothelial cells. This 9 bp sequence, 5′-CTACGTGCT-3′ is identical to the binding site for HIF-1 identified in human erythropoietin gene. In addition, Palmer *et al*. [166] showed that an induced nuclear factor binds to an oligonucleotide containing the putative HIF-1 binding site and mutation of the site eliminates the binding activity. Comparison of the sequences around the HIF-1 site present in the 5′-flanking region of the type II NOS gene and the 3′ enhancer of the erythropoietin gene show a region of similarity 10 bp downstream of the HIF-1 site. The 5 bp sequence, 5′-CACTG-3′ is found to be similar to site 2, 5′-CACAG-3′ of the erythropoietin gene enhancer. Palmer *et al*. [166] proposed that possibly the 5′-CACTG-3′ in the NOS gene could be involved in the hypoxic response. It has been reported that activating transcription factor (ATF-1) and adenosine 3′,5′ cyclic monophosphate response element binding protein (CREB)-1 constitutively bind to the HIF-1 consensus sequence [167] although it is unclear what happens during hypoxia. NO has been shown to modulate the HIF-1 response under hypoxic conditions and also function as a HIF-1 inducer [168]. It is probable that other transcription factors may also be present which may be close to or overlap with the HIF-1 consensus sequence and may be involved in regulation of basal type II NOS gene expression in pulmonary endothelium [166]. The published sequence of the human type II NOS gene does not contain the known HIF-1 binding site and its role is also not known. It is probable that the site may be present upstream from the known published sequence and may be involved in hypoxic regulation of type II NOS gene or else the human type II NOS may not be regulated by hypoxia via HIF-1 [166]. Putative binding sites for other transcription factors like AP-1 and NFκB are also found to be present in the human NOS II gene and may be involved in regulation of this gene under hypoxia.

Role of NO

It is being increasingly recognized that nitric oxide (NO) is involved in many physiological processes viz. neurotransmission in the nervous system, control of blood pressure and immune response [169] as well as modulation of breathing during hypoxia [165]. Generally, under action of short term or moderate stressor increased NO production is observed which corresponds to the stage of mobilization in response to appropriate stress reaction; while decreased NO production is observed in application of long term or detrimental impact of stressors corresponding to the stage of exhaustion in excessive stress reaction [3]. NO is a short-lived, small reactive inorganic molecule, a paramagnetic gas with an unpaired electron, thus making NO a free radical; however, unlike other free radicals, it does not dimerize or dismutate to gain or lose an electron. It reacts rapidly with molecular oxygen and oxygen radicals generating highly reactive compounds like peroxynitrite ($OONO^-$) [170]. The reaction is complex [143] and requires both molecular oxygen and heme iron which is reduced from Fe^{3+} to Fe^{2+}. Endothelial cells respond to nitric oxide (NO) through activation of soluble guanylyl cyclase (sGC) by binding of NO to the heme moeity of sGC [171]. This induces conformational changes that increases its activity, leading to the conversion of GTP to cGMP. cGMP is intimately involved in many signal transduction pathways targeting cGMP-dependent protein kinase, cGMP-gated cation channels and cGMP-upregulated phosphodiesterases [171]. Hypoxia induced pulmonary endothelin-1 expression seems to remain unaltered by nitric oxide [172].

Maladaptive response to HA hypoxia: Altitude illness

Altitude illness is a set of syndromes due to hypoxic injury and/or maladaptive physiological changes occurring at HA generally above 2500 m. Altitude illness is generally divided into three syndromes: acute mountain sickness (AMS), high altitude pulmonary edema (HAPE), and high altitude cerebral edema (HACE). These syndromes are believed to be connected pathophysiologically, but just why cerebral symptoms predominate in some people and pulmonary symptoms predominate in others is not known.

People who rapidly ascend to HA experience symptoms of AMS and the rate of ascent to altitude is probably an important determining factor. Some may have a predisposition to altitude illness, suffering with each visit, though many will not have a recurrence. Predicting who will get sick is not possible. Physiological events associated with pathophysiology of established AMS include hypoventilation [173], im-

paired gas exchange [174], fluid retention and redistribution [175] and increased sympathetic drive [176]. Free O_2 radical damage is increased at HA [177] and this may probably play a role in AMS. It is suggested that an interplay between brain water content, brain blood volume and intracranial dynamics can determine the predisposition to AMS [178].

A more severe, potentially life threatening form of altitude illness is High Altitude Pulmonary Edema (HAPE). Altitude, speed, mode of ascent and individual susceptibility are the most important determinants for occurrence of HAPE [179]. Factors thought to be responsible for development of HAPE are increased pulmonary vasoconstriction which may possibly be related to endothelial dysfunction and sympathetic overactivation [180–182] as well as a defect in transepithelial sodium and water transport [183]. Cultured alveolar cells exposed to severe hypoxia have been shown to upregulate the activity and expression of the glucose transporter GLUT-1 mRNA and downregulate activity and expression of ion transport proteins such as Na^+ channels and Na^+-K^+-ATPase (involved in transport of Na^+ from alveolar to interstitial spaces facilitating resorption of alveolar fluid) [184]. Clericii and Matthay [184] suggested that hypoxia induced down regulation of Na^+ transport proteins *in vitro* may have important pathophysiological implications *in vivo.* Decreased expression and activity of Na^+ transport proteins in ATII cells may slow the rate of reabsorption of alveolar edema. Suzuki *et al*. [185] reported a decrease in alveolar clearance in rats which were exposed to hypoxia. It has been suggested that augmented alveolar flooding, related to exaggerated pulmonary vasoconstriction with the possible conjunction of a defect in alveolar fluid clearance could be the major pathogenic factors in HAPE coupled with a defect in pulmonary NO production [182]. Pulmonary exhaled NO is approximately 30% lower in HAPE-prone than in control subjects. Duplain *et al*. [182] proposed that in HAPE-prone subjects, a defect in pulmonary epithelial NO synthesis may contribute to exaggerated hypoxic pulmonary vasoconstriction and in turn to pulmonary edema. It is also probable that NADPH oxidase generated vasoconstrictive ROS under hypoxia may be a possible mechanism by which pulmonary vasoconstriction could be triggered [186]. Hypoxia has been shown to evoke an inflammatory response in vascular tissue and human peripheral blood mononuclear cells as determined by augmentation of expression of inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukins (IL-1, IL-6, IL-8), inducible nitric oxide synthase, and adhesion molecules (83, 84, 187). These inflammatory processes are likely to be involved in the pathogenesis of HAPE and HACE. Elevated levels of IL6 and C-reactive protein (CRP) in serum [188] and IL6 in bronchoalveolar fluid [189] have been measured in HAPE subjects. Pavlicek *et al*. [190], however, presented evidence against a clinically relevant inflammation in the initial phase of exposure to hypoxia in HAPE susceptible people; although, acute-phase proteins complement (C3), alpha1-antitrypsin (alpha1AT) were shown to be mildly induced.

Genetic determinant of hypoxia response and individual susceptibility to HA disease

Several lines of evidences suggest that control of pulmonary circulation and ventilation at HA may be genetically controlled [191]. Hypoxic ventilatory response (HVR) at low altitude shows considerable variation both within and among individuals suggesting a role of genetic contribution to ventilatory control which may determine ventilation and function at high altitude. Risk factors for AMS include low ventilatory and cardiac response to hypoxia suggesting genetic contribution. Subjects with low HVR measured at sea level are shown to be predisposed to development of AMS [192]. A correlation is also seen between low arterial saturation (SaO_2) and development of AMS although correlation between SaO_2 and HVR has not been demonstrated [193].

The hypoxic pulmonary vascular response has been viewed as a key feature of HAPE. High pulmonary artery pressures (PAP) have been recorded in sufferers of HAPE [194] and in those susceptible [195–197] although it is not a ubiquitous finding and cannot provide reliable forewarning of HAPE [198]. Presence of variable PAP responses to hypoxia between individuals suggest the possibility of genetic polymorphism. Under normal circumstances, a genetic polymorphism (which is a subtle variation in a gene and produces minor changes in function) would pass unnoticed with no observable effect, but changes in environment may elicit an unexpected result, for better or worse. An attenuated HVR has also been observed in some sufferers of HAPE and has been proposed as a potential mechanism of pathogenesis; some subjects, however, show normal or even heightened HVR but still develop HAPE, whereas others demonstrate attenuated responses and still remain asymptomatic [173, 199–201]. There does not appear to be any definite pattern, but the observed attenuation of the HVR in most HAPE susceptible subjects implies differences in carotid body chemosensitivity. Angiotensin Converting Enzyme (ACE) gene polymorphism and susceptibility to HAPE have also been noted [202].

Genetic determinant of hypoxic pulmonary vasoconstrictor response and the ventilatory response to hypoxia may influence the characteristics of the hypoxic sensor, ion channels and/or effector properties of carotid body type I cells. O_2 sensing by carotid and non-carotid chemoreceptors appears to be controlled by distinct genetic mechanisms, because partial deficiency of the HIF-1α eliminates responses mediated by the former but not the latter [165]. Genes and

the mechanisms through which these genes alter the hypoxic pulmonary vasoconstriction or ventilatory response, however, remain to be elucidated.

It is probable that development of HA malady in some individuals may depend on the presence of genetically controlled predisposing factors interacting with environmentally determined precipitating factors. The search for potential causative genes is, therefore, rather complicated and an unknown number of genes and their polymorphisms may be involved in etiology of HA malady. Emerging data indicate that a small number of common variants in the coding regions of genes explain the vast majority of human protein polymorphism. Humans around the world carry pretty much the same genetic material (99.9%) except for minor variations (0.1%). It is these variations that cause diversity among the population as well as susceptibility to diseases. On a genomic level, the most common type of such genetic variation is the single nucleotide polymorphism, or SNP occurring approximately once every 1000 base pairs [203, 204]. Located in both the coding and non-coding regions of the genome, SNPs have been found upstream and downstream of coding regions, or within a gene itself. Once identified and mapped, these genomic landmarks may be essential for investigating genetic factors associated with complex traits encoded by multiple genes. Many of the SNPs in the human genome are likely to do nothing, others are likely to be the key changes that make us individual, while others may affect our susceptibility to many of the common diseases [205]. Identifying the SNPs and other types of polymorphisms and correlating the risk of HA malady with the presence of specific SNPs and/or polymorphisms would be a worthwhile effort in developing biomarkers for susceptible individuals.

Future perspective

The molecular details of hypoxia-induced cellular responses and role of genes can be understood by the temporal, developmental, topographical, histological and physiological pattern in which the gene is expressed. Correlation of gene expression to various biochemical pathways can be demonstrated by the 'chip' technology [206] which is a powerful new approach for simultaneously monitoring the relative expression of a large number of genes in a quantitative fashion. It may thus provide a useful link between gene expression and HA clinical diagnosis. Knowledge of highly selective gene expression, as well as sequence homology to a known gene family, could provide a convenient shortcut for implicating a target in a given pathway or disease. What is of interest for most medical purposes is to know how each gene varies from one person to another and how those variations influence an individual's susceptibility to environmental stress.

All the experiences of the body are ultimately manifest in its protein content. Post-translational modification of proteins plays a pivotal role in modulating the function of many proteins which are not directly coded by genes. As a consequence, the information from a single gene can encode as many as 50 different protein species. While human genome is estimated to have approximately 30000–40000 genes, it potentially encodes 100,000 different proteins. Alternative RNA splicing and post-translational modification may increase this number to up to 5 million proteins or protein fragments. Study of proteomics will provide a complete and accurate profile of protein's abundance or its final structure and state of activity under conditions of high altitude hypoxia. The combination of genomics and proteomics will ultimately play a major role in biomedical research in solving the as yet unresolved issues.

Conclusion

HA hypoxia involves a range of diverse responses which occur at different organizational levels in the body. At the level of organism there is an increase in alveolar ventilation involving interaction of chemoreceptors, the respiratory control centers in the medulla and the respiratory muscles and the lung/chest wall systems; at the tissue level there is pulmonary vascular smooth muscles constriction leading to hypoxic pulmonary vasoconstriction thus optimizing blood flow to alveolar tissues. In coronary and cerebral vessels there is vasodilation that increases the perfusion of blood to the O_2 deprived tissues; at the cellular level there is down regulation in metabolic rate, release of neurotransmitters by the glomus cells of the carotid body, secretion of erythropoietin hormone by kidney and liver cells and release of vascular growth factors by parenchymal cells in many tissues, increase in intracellular calcium, opening of K^+ channels in nerve cells, activation of number of intracellular kinases; at the molecular level there is an induced expression of several genes involved in erythropoiesis, angiogenesis, tissue remodeling, vasomotor control, neurotransmitter release and glucose metabolism through activation of transcription factors.

There is an intricate interplay of biochemical pathways in response to high altitude hypoxia, which cause changes at the physiological, cellular and molecular levels. Cellular and molecular mechanisms of many of the responses are yet to be elucidated. Added to the interplay is also the possibility of genetic polymorphism and protein changes for adaptation to environmental influences, which further allow variability in the activity of the pathway. Expression profiling and proteomics will further enhance our understanding of these interactions. It may be that one is close to the precise combination of genetic and protein factors that unlock the prob-

lem of what goes on under high altitude hypoxic stress and who will cope at high altitude. Furthermore, knowledge gained in the area of mountain medicine can also be correlated in the biomedical field involving hypoxic situations like oncology, embryogenesis and cardio-pulmonary disorders and vice versa.

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