Abstract

High altitude is characterized by extreme environmental conditions such as low atmospheric pressure, low partial pressure of oxygen, low temperature, and high ultraviolet radiation. It has been well-documented that the hypobaric hypoxic environment at high altitude causes a number of disorders in sojourners (lowland population) while at the same time the permanent residents of high altitude remain hale and hearty for the same disorders. The present thesis work explores the genetics and molecular basis of adaptation and mal-adaptation in the permanent residents and the sojourners. The thesis work has been structured into 5 chapters. In Chapter 1, review of literature pertinent to the work performed in the thesis has been presented. Among many high altitude associated disorders, our primary focus remained on a rare, fatal (if not treated) and morbid respiratory illness namely, high altitude pulmonary edema (HAPE). HAPE is a life-threatening form of non-cardiogenic pulmonary edema that occurs in unacclimatized sojourners on exertion. It is caused by acute exposure to low partial pressure of oxygen at high altitude. Speed of ascent and altitude attained are the key determinants in occurrence of this disorder. HAPE is characterised by exaggerated hypoxic pulmonary vasoconstriction leading to an abnormal build-up of fluid in the lungs and vascular leakage through overperfusion, stress failure, or both. Although, many pathways, such as oxidative stress, oxygen-sensing, vascular homeostasis, sympathetic nervous system are reported to be involved in the pathophysiology of HAPE. However, the primary signalling entities, such as, kinases have not been studied in detail except its one of the constituents namely, tyrosine kinases. In contrast, only few studies have described the role of serine-threonine kinases under hypobaric hypoxic conditions. The present study thus focuses on the role of three major serine-threonine kinases, i.e., rho-associated coiled-coil kinase isoform 2 (ROCK2), myosin light chain kinase (MYLK) and c-jun N-terminal kinase 1 (JNK1), in the signalling of sympathetic nervous system via alpha-1 adrenergic receptor system under stress (hypobaric hypoxia) conditions of high altitude. Since, biology is complex and in order to understand a pathway in totality many molecules need to be studied hence, apart from the genetic screening and
expression of the three kinases, six other genes associated with the concerned signalling pathway were also studied namely, i) TH (tyrosine hydroxylase), ii) G-protein subunits: iia) GNA11 (guanine nucleotide-binding protein (G protein), alpha II (Gq class)) and iib) GNB3 (guanine nucleotide-binding protein (G protein), beta polypeptide 3). iii) alpha1-adrenergic receptor isoforms: iiia) ADRA1A (adrenoceptor alpha 1A), iiib) ADRA1B (adrenoceptor alpha 1B), and iiic) ADRA1D (adrenoceptor alpha 1D). In addition, eight circulatory biomarkers namely, epinephrine, norepinephrine, dopamine, TH, transforming growth factor beta 1 (TGFβ1), tumour necrosis factor alpha (TNFα), platelet-derived growth factor beta beta (PDGF-ββ) and C-reactive protein (CRP) were also studied. The study groups were composed of HAPE patients (HAPE-p), HAPE controls (HAPE-c) and Highlanders (HLs). HAPE patients (HAPE-p) were the sojourner/lowlanders, who acquired HAPE upon ascent to high altitude (~3500 meters) on first exposure. HAPE controls (HAPE-c) were the sojourners, who did not acquire HAPE upon ascent to high altitude (~3500 meters) under similar conditions as experienced by HAPE-p. While highlanders (HLs: >3500 metres) were healthy natives of the high altitude region, residing for generations. In addition, animal study was performed to validate few of the results. The following text describes each specific experiment.

Chapter 2: A biomarker is a measurable indicator of any biological state or condition. Estimating circulatory biomarkers has always remained a mechanism to understand the pathophysiology of any disease. Hence, in this chapter role of 8 circulatory biomarkers namely, epinephrine, norepinephrine, dopamine, TH, TGFβ1, TNFα and CRP in HAPE pathophysiology and adaptation was studied. The biomarkers were measured in human blood plasma of the three study groups. Stress activated sympathetic nervous system releases catecholamines (epinephrine, norepinephrine and dopamine) via the enzyme TH. Catecholamines activate G_{q/11}-coupled alpha1-adrenergic receptors on the smooth muscle cells, thereby inducing a membrane bound enzyme phospholipase-C that leads to an increase in intracellular Ca^{2+} concentration through endoplasmic reticulum. Increased calcium ion concentration activates MYLK, which in turn, phosphorylates myosin light chain at serine residue 19 inducing the formation of actin-myosin cross-bridge formation.
thus causing exaggerated vasoconstriction. Likewise, stress activated ROCK2 works in conjunction with MYLK to cause exaggerated vasoconstriction by directly phosphorylating myosin light chains and inhibiting an enzyme called myosin light chain phosphatase that acts in reverse direction to MYLK. ROCK2 also attenuates the synthesis of critical vasodilatory molecule nitric oxide that is involved in HAPE pathophysiology. Stress activated JNK1 (third kinase being studied) decreases voltage-gated potassium channel activity leading to a depolarization wave across the membrane that in effect increases intracellular Ca\textsuperscript{2+} concentration and smooth muscle cell proliferation. Among the other molecules, TGFβ1, TNFα, PDGF-ββ and CRP are the circulatory biomarkers that are induced under stress conditions. TGFβ1, a regulator of collagen synthesis and vascular remodelling in fibroblasts; TNFα, a pleiotropic pro-inflammatory cytokine; PDGF-ββ, a pro-proliferative growth factor and CRP, a pro-inflammatory marker activate both ROCK2 and JNK1 thereby inducing exaggerated smooth muscle cell contraction and proliferation. Results demonstrated that exaggerated sympathetic nervous system activity is a feature of HAPE as evidenced by significantly elevated levels of epinephrine, norepinephrine, dopamine, TH in HAPE-p when compared with both the healthy groups i.e. HAPE-c and HLS (p<0.0001). Moreover, molecules like TGFβ1 and TNFα are also associated with HAPE, as also evidenced by their significantly elevated levels (p<0.0001). On the other hand, less or non-significant p-values between HAPE-c and HLS demonstrated that even though HLS and HAPE-c had small differences in the levels of these circulatory biomarkers, these levels were good enough to supposedly protect against HAPE (p>0.01). CRP was observed to have a protective role as the CRP levels were significantly reduced in HAPE-p when compared with HAPE-c and HLS (p<0.0001). The PDGF-ββ levels did not differ significantly in HAPE-p when compared with the healthy groups i.e. HAPE-c and HLS (p>0.01). Nevertheless, more studies are needed in this field pertaining to these parameters. Together, these biomarkers can serve as biomarkers in HAPE diagnostics, prognostics and even may also work as predisposing markers.

Chapter 3: The genetic basis of HAPE in sojourners and its adaptation in highlanders is poorly understood. There seems to be a genetic basis to HAPE in sojourners, as only a
small portion of the lowland population acquires this disorder, while the majority remains healthy on ascent to high altitude. In contrast, highlanders, the permanent residents of high altitude, remain well adapted to HAPE. Hence, in this chapter, the study was aimed to determine genetic variants that associate with HAPE. In this regard, 3 kinases (ROCK2, MYLK1, JNK1) along with 6 other genes namely, i) TH, ii) GNAI11, iii) GNB3, iv) ADRA1A, v) ADRA1B, and vi) ADRA1D were studied as candidate markers of HAPE and adaptation in order to investigate the pathway in totality. These are the primary signalling entities of stress activated sympathetic nervous system signalling pathway, that in effect, culminates into the progression of exaggerated vasoconstriction and smooth-muscle proliferation, hallmarks of HAPE. Fifty-seven variants across these nine genes were genotyped in HAPE-p (n=225), HAPE-c (n=210) and HILs (n=259), which revealed a significant association of few alleles such as the C allele of ROCK2 SNP, rs10929728 with HAPE (p=0.03) and C, T and A alleles of MYLK SNPs, rs11717814, rs40305, and rs820336, respectively, with both HAPE and adaptation (p<0.02). ROCK2 GGGTTGGT haplotype, a 88kb stretch, was observed to be associated with lower risk of HAPE (p=0.0009). MYLK haplotype GTA, a 7kb stretch, was observed to be associated with higher risk of HAPE (p=0.0006) and lower association with adaptation (p=1E-06), while haplotype GCG, composed of wild-type alleles was observed to be associated with lower risk of HAPE (p=0.001) and higher association with adaptation (p=1E-06). Haplotype-haplotype and gene-gene interactions demonstrated a correlation in the working of ROCK2 and MYLK. The data suggested an association of ROCK2 with HAPE and MYLK with both HAPE and adaptation.

Chapter 4: Gene expression is the most fundamental level at which the genotype of an organism gives rise to the phenotype. In this chapter, transcript expression of nine genes under study namely, ROCK2, MYLK, JNK1, TH, GNAI11, GNB3, ADRA1A, ADRA1B and ADRA1D was measured in human peripheral blood mononuclear cells (PBMCs). Our results demonstrated that there were no significant fold changes in expression for the nine genes under study, but there was a pattern in the expression of the three kinases and TH. The fold differences of up to ~1.4 or above were observed (p<0.05). We assume that increase in the number of samples might help in achieving the level of significance. For
the remaining five genes namely, GNAI1, GNB3, ADRA1A, ADRA1B and ADRA1D the
fold differences were largely between -1.3 to +1.1 (p < 0.05). We attribute these results to
the low expression of these molecules in human PBMCs as these molecules are expressed
highly in tissues.

Chapter 5: In this chapter, an animal study was performed. Since, HAPE is a lung
disorder and human lung tissues were not available due to non permission; moreover, as
the blood levels/expression wasn’t the true indicator of these genes, hence, it became
important to study the three kinases in animal lung tissue. Wistar rats were used as a
model organism. Furthermore, protein level studies were not possible in PBMCs as these
kinases are expressed highly in tissues. It is imperative to study the molecules at protein
level as they exert their final effect in protein form. Apart from lungs, heart, brain and
kidneys are the three other vital organs being affected at high altitude. Thus, we have
studied the three kinases in these tissues as well. Prior to the study, the animals were
divided into 3 groups of 5 animals in each, (a) Control Group: Sacrificed on the first day
(no treatment group), (b) & (c) Group 1 and Group 2, respectively; animals were exposed
to hypobaric hypoxia for 12 hour in a hypobaric hypoxic chamber (Seven Star, New
Delhi, India) at 426 mm Hg, which was equivalent to ~15,000 ft. The humidity in the
chamber was maintained between 40-60%. (b) The animals of Group 1 were sacrificed
immediately upon completion of the experiment, and (c) The animals of Group 2 after the
experiment were followed by an aclimatization period of 12 hours at ambient conditions
(normobaric normoxia). The animals were sacrificed immediately upon completion of the
12 hour normoxic exposure. Tissue sections were stained with different stains to
understand the changes in tissue morphology. The kinases were also studied at transcript
and protein levels in other tissues. Immunohistochemistry and western blot analyses of
the lung tissues were performed to confirm the presence of kinases in various tissues.
Results showed that there was a formation of a large perivascular edema cuff and
collagen deposition in lung tissues upon 12 hour hypobaric hypoxic exposure and a
reduction upon acclimatization to normobaric normoxic conditions. Protein levels and
activities of ROCK2, MYLK, and JNK1 in lungs were elevated after 12 hour hypobaric
hypoxic exposure and the same parameters were observed to be normal upon simulation
to high altitude descent conditions. The gene expression pattern for the three kinases was not significant in lungs among the three study groups (p>0.05). This could be attributed to the difference in transcription and translation rates due to which significant differences were observed at protein levels against non-significant levels at transcript level. As for few mRNA transcribed, a lot more amount of protein is generally translated. The results of the present study have noticeably signified the role of three kinases namely, ROCK2, MYLK, and JNK1 under hypobaric hypoxic conditions as experienced under high altitude settings.