Interplay of lung cytoskeletal re-modeling, energy metabolism and inflammation during extended exposures to lowered pO2

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Abstract

Extended exposure to low pO2 has multiple effects on signaling cascades. Despite multiple exploratory studies, specific studies elucidating the response of lung and plasma proteome in context of surviving low pO2 exposures are lacking. In this study, we simulated low pO2(PB=40 kPa; 7620 m) exposure in male Sprague-Dawley (SD) rats for 3, 7 and 14 days. Redox stress assays and proteomics were performed using lung and plasma followed by protein network analyses. We observed that redox homeostasis was achieved after day 3 of exposure. Lung proteome revealed cytoskeletal processes were the most significant with STAT3 acting as upstream regulator. Plasma proteome revealed a focus on lipid metabolism derived inflammatory processes. Overall, during prolonged low pO2 exposure, particularly those involving slowly decreasing pressures, redox homeostasis is achieved but energy metabolism is perturbed and this leads to an immune/inflammatory signaling impetus after 3rd day of exposure. We found that an interplay of lung cytoskeletal elements, systemic energy metabolism and inflammatory proteins aid in achieving redox homeostasis and surviving extended low pO2 exposures. Qualitative perturbations to cytoskeletal stability and innate immunity/inflammation were also observed during extended low pO2 exposure in humans exposed to 14,000 ft for 7, 14 and 21 days.

Introduction

Low pO_2 conditions, also termed environmental hypoxia, are expressly encountered as one ascends to altitude. In a previous study, we delineated the effects of varying levels of pO_2 during acute exposure of 24 h on the lung and plasma proteomes. We observed that the lung's redox homeostasis, cytoskeletal integrity and energy homeostasis was greatly perturbed at very high altitude zone (simulated) when the ascent rate was very high. In addition, we also observed global proteome responding to these perturbations and affirming systemic redox and energy homeostasis (Paul, Gangwar, Bhargava, & Ahmad, 2018). However, the effects of extended exposure to low pO_2 conditions at varying time points remain unknown. Hence, we needed to investigate the effects of increasing the duration of low pO_2. We chose the lowest survivable pO_2 (8.19 kPa) corresponding to P_B 40 kPa which is the atmospheric pressure encountered at 25,000 ft (7620 m; beginning of death zone altitude zone).

In this study, we focus on the effects of increasing durations of low pO_2 exposure (8.19 kPa) on the lung and plasma proteomes. Previous studies regarding extended low pO_2(environmental hypoxia) exposure have elucidated multiple facets of exposure to chronic environmental hypoxia at high altitude. A pioneering human study on arterial pH and hemoglobin affinity during extended environmental hypoxia exposure was conducted by Chiodi in 1957 (Chiodi, 1957). Mazzeo and co-workers have reported a higher arterial norepinephrine and epinephrine concentration after 21 days at Pikes Peak (˜P_B 61 kPa; pO_2 12.81 kPa) in human subjects (Mazzeo et al., 1991). Other studies on endocrine system during extended low pO_2 exposure report a suppressive effect of endocrine mediators to facilitate maximal energy efficiency even at the cost of better oxygen delivery (Barnholt et al., 2006; Chaiban, Bitar, & Azar, 2008). Weight loss is a highly studied

phenomenon during chronic low pO_2 exposure and is attributed in part to hormones like leptin (Boyer & Blume, 1984; Butterfield et al., 1992; Hamad & Travis, 2006; Surks, Chinn, & Matoush, 1966; Tschöp & Morrison, 2001; Tschöp, Strasburger, Hartmann, Biollaz, & Bärtsch, 1998). Physiological features like heartbeat interval distributions were reported to be affected by chronic environmental hypoxia (Meyer et al., 1998). Ostadal and Kolar have extensively reviewed the cardiac adaptations during extended low pO_2 exposures, particularly the underlying molecular mechanisms (Ostadal & Kolar, 2007). An exquisite review focused on cardiac metabolic perturbations during extended exposure to low pO_2 was authored by Essop (Essop, 2007). Even in the early 80s, researchers had implicated energy efficiency with survival during chronic low pO_2 exposures owing to increased energy expenditure and reduced caloric intake (Durand, 1982). A greater emphasis was laid on the muscle tissue due to their energy intensive nature during extended exposure to low pO_2. Cerretelli and Hoppeler provide an early review of metabolic consequences of extended environmental hypoxia exposure on the muscle (Cerretelli & Hoppeler, 1996). Early reports stated changes in muscle capillary geometry during chronic environmental hypoxia in rats (Poole & Mathieu-Costello, 1989). The same authors also elucidate the effects of chronic environmental hypoxia on deer mouse muscle fiber size (Mathieu-Costello, Poole, & Logemann, 1989). Human muscle studies revealed a lower mitochondrial density and fiber size with unchanged capillary network during mountaineering expeditions which involved low pO_2 exposures for extended time periods (Hoppeler et al., 1990). Similar studies revealed an overall shift to anaerobic metabolism in muscles (Howald et al., 1990). Muscle metabolism selectively utilizing free fatty acids instead of glycogen reserves after extended exposure to low pO_2 was reported by Young and colleagues (Young, Evans, Cymerman, Pandolf, & Knapik, 1981). The influence of exercise on muscle during high altitude ascent and extended environmental hypoxia exposure has also been discussed by Kayser and co-workers in context of muscle fatigue where they showed that the nervous system had a bigger role to play rather than metabolic intermediates like lactate in causing exhaustion (Kayser, Narici, Binzoni, Grassi, & Cerretelli, 1994). A more recent study focused on the muscle mitochondrial processes during low pO_2exposure (pO_2 9.87 kPa) of up to 66 days and stated that muscle energy utilization and oxidative stress were modulated towards efficient ATP generation (Levett et al., 2011). Radak *et al* have provided a detailed review of oxidative stress during environmental hypoxia (Dosek, Ohno, Acs, Taylor, & Radak, 2007) and linked DNA repair with fatty acid metabolism in human skeletal muscle exposed to environmental hypoxia (Acs et al., 2014). Another study during Caudwell Xtreme Everest expedition concluded that chronic environmental hypoxia exposure caused significant muscle atrophy, however, muscle function remained constant (Edwards et al., 2010).

Lung (Pulmonary) system has also been studied during extended exposures to low pO_2. Few of the earliest publications detail carotid body insensitivity during extended low pO_2exposure in man (Severinghaus, Bainton, & Carcelen, 1966; Weil, Byrne-Quinn, Sodal, Filley, & Grover, 1971). In 1975, Tucker and co-workers implicated lung vascular smooth muscle as determinant of pulmonary hypertension, common during extended low pO_2exposures (Tucker et al., 1975). Later on, mechanical properties of lung as an organ were elucidated (Weil, 1986). Decreased pressor response and increased mast cell density have been reported in rats exposed to longer durations of low pO_2 (McMurtry, Petrun, & Reeves, 1978; Tucker, McMurtry, Alexander, Reeves, & Grover, 1977). Pulmonary hypertension during low pO_2 exposure at high altitude has been reported in numerous studies and said to be a causative factor of HAPE and CMS (Aldashev et al., 2002; Antezana et al., 1998; Ge & Helun, 2001; Maggiorini & Leon-Velarde, 2003; Naeije, De Backer, Vachiery, & De Vuyst, 1996; Rabinovitch, Gamble, Miettinen, & Reid, 1981; Scherrer et al., 1999; Vender, 1994; Yagi, Yamada, Kobayashi, & Sekiguchi, 1990). Molecular aspects of the hypoxic lung have also been investigated. HO-1, VEGF and Rho kinase based signaling events have been implicated in chronic hypoxic lung (Gao et al., 2007; Tuder, Flook, & Voelkel, 1995). However, rat lung proteome analysis focused on extended exposure to low pO_2 are rare (Ohata et al., 2013). Only recently, studies on the effects of acute low pO_2exposure on rat lung proteome have been reported (Ahmad et al., 2015). Thus, this study focusing on extended low pO_2 exposure induced lung proteome alterations in rat was explorative in nature. Furthermore, we also investigated changes in the plasma proteome of same animals so as to find the interlinks between the two proteomes. This was done to further facilitate their translational application during later investigations. This study elucidated many crucial aspects of lung and plasma proteome during extended exposure to low pO_2. One of the key findings was that unlike redox homeostasis being a central process during acute exposure (Paul et al.,

2018), extended exposure caused cytoskeletal re-arrangements to dominate in lungs while plasma proteome was dominated by proteins involved in metabolic and immune processes. An overall view that emerges from this study is the cytoskeletal remodeling that occurs in the lung signals the systemic response manifested in energy metabolism and inflammation signaling processes. A common low pO_2 mediator between lung and plasma is STAT3 (Signal transducer and activator of transcription 3) (Paul et al., 2018). In an attempt to gather qualitative insights in humans undergoing extended exposure to low pO_2conditions, we performed ELISA based validations of Alpha-1-antitrypsin, cofilin-1 and S100A8 in humans exposed to 14,000 ft (˜pO_2 12.81 kPa) for 7, 30 and 120 days. The selection of these proteins was from the protein datasets of rats, with the focus being that each of the proteins represent the processes found perturbed in rat, i.e. lung cytoskeletal re-modeling, and its derived innate immune/inflammatory signaling.

Overall, we observed that extended exposure to low pO_2perturbed lipid metabolism due to redox stress in pursuit of redox homeostasis. The disturbed lipid metabolism led to inflammatory processes being activated. STAT3 played an important upstream regulatory role which may be specifically evaluated in future investigations.

Materials and methods

Study design

Ethics statement

All animal experiments were undertaken as per the ARRIVE guidelines after approval from Institutional Ethics Committee, DIPAS. Informed written consent was obtained from all human volunteers prior to blood collection. The experimental procedures for human volunteers were approved by the Institutional Ethics Committee, DIPAS in accordance with the Declaration of Helsinki.

Extended exposure to low pO_2

A custom designed pO_2 modulation chamber (7 star systems, Delhi, India) was used. A constant temperature (25±5 @C) and relative humidity (50±5%) were maintained in the simulation chamber during experiments. Airflow of 2 L/min was provided to Sprague-Dawley (SD) rats undergoing hypobaric hypoxia exposure. The animals were provisioned with food and water *ad libitum* during the entire duration of low pO_2 exposure. Age and gender matched male SD rats were exposed to either normoxia (n=5) or low pO_2 of 8.19 kPa (equivalent altitude: 7620 m; simulated). Exposure lasted for 3 days (HD3), 7 days (HD7) and 14 days (HD14) (n=5 per group) of stay in the pO_2 chamber. The rate of decrease of P_B was ˜4.1 kPa/min. Exposure to P_B 40 kPa (pO_2 8.19 kPa) began after 12 h each at P_B 71 kPa and P_B 59 kPa. The purpose of such a slow and graded low pO_2 exposure was to minimize mortality of animals and mimic real-world conditions of altitude ascent. After the completion of normal and low pO_2 exposures, the animals were sacrificed and their lungs and plasma collected. Further, quantitative proteomics investigations, biochemical assays, assessment of redox specific transcripts and validations using immuno-chemical methods were performed on these. All animals were sacrificed using approved procedures to minimize pain and suffering immediately after the exposure. Our study design was initially approved by the Institute's Animal Ethics Committee (Fig. 1).

Housing conditions of experimental animals

Male SD rats were used for the animal experiments. All animals used were housed in polypropylene cages (3-4 animals per cage) with sterilized straw. Hydration and nutrition were provided *ad libitum* . Temperature of the housing complex was always kept around 25 ^{o}C±3 ^{o}C with relative humidity at 50-55%. Animals used for a particular study were always from the same cohort. Light/dark cycles were of 12 h each. All care was taken to avoid any discomfort or pain during experimentation. All studies were carried out in accordance with the Institutional Animal Ethics Committee's regulations which are based on the NIH Guidelines for care and use of animals during experimental procedures.

Collection of tissues and blood samples and extraction of plasma in Rats

The site of blood collection was the retro-orbital vein. Rats underwent general anesthesia using Ketamine:Xylazine recommended dose (i.p.) prior to blood extraction. The blood was collected in EDTA/citrate lined tubes. These tubes were then centrifuged for 15 min at 3500 rpm. PI cocktail (Cat. no. P8340, Sigma, USA) was added immediately thereafter. After the sacrifice of rats using lethal dose of ketamine:xylazine, their lungs were sectioned and rinsed in cold PBS. All plasma and lung tissue samples were snap frozen using liquid nitrogen and stored in -80°C.

Lung tissue, after de-freezing to room temperature, was homogenized in RIPA buffer (1:10 w/v) and sonicated (three times for 5 seconds each) briefly. Then the homogenate was centrifuged (15,000 g ; 20 min) at 4°C and the clear supernatant was used for assays.

Collection of human blood and extraction of plasma

Human volunteers (male; mean weight: 65 kg; mean age: 24 y) were exposed to high altitude (4267 m; 14,000 ft; pO_2 12.81 kPa) for 7 days (HAD7), 30 days (HAD30) and 120 days (HAD120) subsequent to their stay in normoxic conditions (Baseline). All were reported to be acclimatized and free of altitude illnesses. Obesity, hypertension, addiction, lung diseases and other diseases resulted in exclusion from study. Blood samples (2 ml) were collected from the antecubital vein while the volunteers (n=3 per group) lay prone. The sample collected was subsequently centrifuged (3500 rpm; 15 mins) and plasma collected in fresh tubes. Protease inhibitor (PI) cocktail (Cat. no. P8340, Sigma, USA) was immediately added to all collected samples and tubes stored at -80 ºC.

Quantitative proteomics using LC-MS/MS

Sample preparation

The plasma/lung tissue homogenate were pelleted using lyophilizer. The lyophilized sample was then re-suspended in lysis buffer. Samples were again centrifuged and both supernatant and lysate separately retained. Pellet was further treated with ToPI protein isolation buffer (K-0011) and centrifuged. The lysate was transferred back into tube containing the retained supernatant. Protein estimation of this total lysate was carried out using ToPA Bradford Protein Assay kit. 100 µg protein per sample were processed to remove reducing, alkylating and interfering substances from the sample before MS analysis. Overnight trypsin digestion was then performed on the sample. iTRAQ reagents were added to the digested peptides for labeling of samples. SCX fractionation of each sample was done. Elution was performed at 75 mM, 150 mM, 450 mM ammonium acetate for each sample. The individual samples were collected and analyzed by nano-LC-MS/MS and combined for MudPIT.

LC-MS/MS

Samples were desalted using ZipTip. Then, samples were lyophilized and subsequently re-suspended in appropriate mobile phase for LC-MS/MS. Elution of peptides was done using linear acetonitrile gradient (5 to 45%) over 180 minutes and immediately followed by high and low organic washes lasting 20 minutes. Samples were injected into an LTQ XL mass spectrometer (Thermo Scientific) using a nano-spray source with 1.8kV spray voltage and temperature of 180 ºC in ion transfer capillary. The Top 5 data dependent method was used for a full MS scan (m/z 400-1500) followed by MS/MS scan (5 most abundant ions). Ratio >1.5 was considered up-regulated, ratio <0.67 was considered downregulated and ratio between 1.5 – 0.67 were considered non-significant. 100-fold was the maximal allowed expression ratio.

Immunoblotting

Both lung tissue homogenate and plasma samples were immunoblotted. Using Bradford assay, sample volume containing 25 µg protein was estimated. Sample volume containing 25 µg protein was separated on 10% SDS-PAGE gel and then transferred onto nitrocellulose/PVDF membrane. 5% skim milk in PBS-0.1% Tween-20 (PBST) was used to block the membrane at 4 °C overnight. The membrane was then washed with PBST thrice (10 min each) followed by sequential incubation with given primary and secondary antibodies for 2 h at room temperature. Secondary antibody was removed and membrane was again washed thrice with PBST.

The membrane was then developed using chemiluminescent peroxidase substrate (Cat. no. CPS1300, Sigma, USA). ImageJ (http://rsbweb.nih.gov/ij/) was used for densitometric analyses of the autoradiograms. 1D SDS-PAGE gels were silver-stained and served as loading controls for both lung tissue homogenate and plasma samples. The following primary antibodies were used for immunoblotting: CLIC5 (Cat. # PA5-41047; Thermo Fisher, USA); GAPDH (Cat. # 21612, SAB, USA); Trx (Cat. # 46892-2, Santa Cruz, UK); RXR (Cat. # 33481, SAB, USA); MDH1 (Cat. # bs3396R, Bioss, USA); HIF-1a (Cat. # NB100105, Novus Biologicals, USA); STAT3 (Cat. # ab76315; Abcam, USA); GPx3 (Cat. # NBP1-06398, Novus Biologicals, USA); Plasminogen (Cat. # ab154560, Abcam, USA); C3 (Cat. # WH0000718M1, Sigma, USA); C4b (Cat. # Ab66791, Abcam, USA); Ttr (Cat. # MAB10762, Merck, USA); Apo A1 (Cat. # sc-30089, Santa Cruz, UK) and Apo H (Cat. # bs-1570R, Bioss, USA).

ELISA

ELISA was performed as per manufacturers' instructions for the proteins alpha-1-antitrypsin (Cat. no. CSB-E11719h, Cusabio, USA), cofilin-1 (Cat. no. CSB-EL005280HU, Cusabio, USA) and S100A8 (Cat. no. CSB-E11833h, Cusabio, USA) using human plasma.

Biological network and pathway Analysis

IPA (Ingenuity Pathway Analysis; Ingenuity Systems; Redwood City, CA www.qiagen.com/ingenuity) analysis (using trial version) was done on all expression value containing protein datasets. Uniprot IDs and respective gene names of proteins served as identifiers. IPA mapped protein IDs were used for core analysis. A threshold of 1.3 and p-value$<$0.05 (using in-built Fisher's exact test) was constant throughout IPA analysis. Ingenuity Pathway Analysis Knowledge Base was the database against which major canonical pathways and protein networks were identified from the experimental dataset. The analysis parameters were specific for the organism and tissue used. IPA also predicted upstream regulator proteins for experimental datasets. IPA based analysis revealed the most significant canonical pathways, upstream regulator proteins, protein-protein networks and protein-process networks.

Gene Ontology (GO) Analysis

Gene Ontology Consortium Enrichment Analysis Tool (PANTHER Overrepresentation Test; Release 20160715) was used for all GO results presented in this thesis. Top significant pathways with p$<$0.05 only were shown. GO represented molecular function, biological processes and cellular compartment/localization for the given protein datasets which were searched against Gene Ontology database (released on 08-22-2016) for specific organism.

Statistical analyses

Graphpad Prism (version 7) was used to interpret experimental data and for its meaningful representation graphically. Statistical analyses of the data were also performed. 1-way ANOVA followed by Tukey's multiple-comparison test/Bonferroni's multiple comparison test was used. Significance was set at p$<$0.05 for animal experiments and p$<$0.01 for human studies. Results were represented as Mean$\pm$SEM (Std error of mean) for animal studies and human studies. For each study, data from three biological replicates were used. All comparisons are Normoxia (Baseline) vs. other groups.

For LC-MS analysis, Target decoy PSM validator algorithm was used in all database searches and an FDR of 0.0098 was calculated by Proteome Discoverer.

Results

Cytoskeletal re-modeling as predominant molecular event in lung during hypoxia

Redox homeostasis remained unperturbed past 3rd day of low pO_2 exposure

Due to our previous study re-affirming redox stress as the principal component of environmental hypoxia (Paul et al., 2018), we began with a biochemical investigation of the lung to determine redox parameters. ROS (Fig. S1a, *Supplementary information*) and TBARS (Fig. S1b,*Supplementary information*) levels tend

to increase significantly at HD3. In HD7 and HD14, there is near restoration of ROS and TBARS levels to those observed in Normoxia group. NOx levels were also measured. NOx levels were minimally up-regulated across HD3-HD14 relative to Normoxia (Fig. S1c).

As the next logical step, we investigated the antioxidant defenses of the lung during hypoxia exposure. TAC (total antioxidant capacity) was nearly twice in HD3 as compared to Normoxia. However, in HD7 and HD14, TAC levels declined successively but remained higher than Normoxia (Fig. S1d, *Supplementary information*). Interestingly, we observed catalase (Fig. S1e, *Supplementary information*) and SOD (superoxide dismutase; Fig. S1f, *Supplementary information*) activity levels to remain nearly constant throughout Normoxia and HD3-HD14. Total glutathione (GSH) concentrations were observed to successively increase at HD3 and HD7 before declining in HD14 to levels similar to Normoxia (Fig. S1g, *Supplementary information*).

We analyzed redox stress specific transcripts in the lung (Fig. S2,*Supplementary information*) to further determine the response to chronic environmental hypoxia. The hierarchical clustergram did not provide any clear directionality as to which groups have the overall highest or lowest transcripts' expression. Almost all transcripts had similar expression across groups. This indicated that redox stress was minimized after the third day of simulated altitude exposure.

Proteomic investigations revealed extensive perturbations in proteins associated with cytoskeletal processes

Thus, we performed iTRAQ-LC-MS/MS based proteomics investigation. In HD3, out of a total of 116 identified proteins, 47 were down-regulated and 5 were up-regulated. In HD7, out of a total of 119 identified proteins, 25 were down-regulated and 16 were up-regulated. In HD14, out of 116 identified proteins, 32 were down-regulated and 11 were up-regulated (Fig. 2a). Molecular and cellular functions that were dominant across all groups were Cellular movement, Cellular assembly and organization and Cell-to-cell signaling and interaction/Cellular function and maintenance (HD7) (Fig. 2b). We also observed that for a majority of proteins in the lung, their overall state of either up- or down- regulation continued to be similar across HD3, HD7 and HD14 as compared to Normoxia in the hierarchical clustergram (Fig. 2c & *Table S1, Supplementary information*). Across groups, a larger number of down-regulated proteins indicates a trend of energy efficiency by down-regulating cell signaling, protein turnover and metabolic activities of the lung tissues. Most notable were increased hemoglobin subunits and carbonic anhydrase levels at HD7, indicating a major signaling impetus towards hematopoiesis and respiratory acidosis (Table S1, *Supplementary information*). Upon IPA analysis of the lung proteome, we observed the top canonical pathways to be Remodeling of epithelial adherens junctions, signaling cytoskeleton signaling, Clathrin-mediated endocytosis signaling and Epithelial adherens junction signaling across HD3 and HD7. Among these, only Remodeling of epithelial adherens and Actin cytoskeleton signaling show directionality in terms of significant z-scores. In HD14, Signaling by Rho-family GTPases replaces Epithelial adherens junction signaling among the top four canonical pathways while also showing directionality (down-regulation) (Fig. 2d and Fig. S3, *Supplementary information*).

The overall directionality of Remodeling of epithelial adherens junctions was neither up nor down-regulated. Thus, no further analysis of the pathway was carried out. Upon detailed investigation of the Actin cytoskeleton signaling pathway with expression levels overlaid from the dataset (Fig. 3a), we observed multiple perturbed cytoskeletal elements involved in processes related to actin polymerization, cytoskeleton re-organization, formation of adherens complex and focal complex assembly. We also assessed the key proteins involved in this canonical pathway across groups. Almost all the cytoskeletal elements were down-regulated in HD3, up-regulated in HD7 with either down-regulation or normalization observed in HD14.

Using IPA, an integrated network of interlinked proteins involved in different significant canonical pathways (Fig. 2d) as well as their upstream regulators was created. This network was then overlaid with protein expression data from the lung proteome (Fig. 3b). We observed STAT3 to be the main upstream protein. Based on the interactions observed among structural proteins (CLIC5, vimentin), antioxidant proteins (thioredoxin), housekeeping proteins (actin, tubulin, GAPDH), metabolism associated proteins (RXR, MDH) we immunoblotted certain proteins known to be associated with these processes in lung tissues (Fig. 3c & d). GAPDH levels were constant across all groups suggesting minimal effects on housekeeping functions. Thio-

redoxin levels were the highest in HD3, normalized in HD7 and again increased in HD14 indicating active antioxidant defense during extended low pO_2 exposure. RXR, involved in lipid metabolism via LXR/RXR signaling, was also observed to up-regulated during low pO_2 exposure especially in HD7 suggesting preferential utilization of lipids during extended exposure to low pO_2. A concomitant and successive decline in malate dehydrogenase (MDH) levels during extended low pO_2exposure marked a shift in energy metabolism away from TCA cycle. The increased impetus towards more efficient utilization of energy is also signified by declined expression of CLIC5 (Chloride Intracellular Channel 5) suggesting inhibition of energy intensive signaling and transport processes during extended low pO_2 exposure. HIF-1a, the master regulator of hypoxia, was observed to have increased expression throughout the extended low pO_2 exposure (particularly HD14). STAT3, identified as a major upstream regulator in our study, slightly decreased in HD3. In HD7 and HD14, STAT3 levels were higher than Normoxia with the highest levels being observed in HD7.

Global proteome analysis suggests a perturbed lipid metabolism and inflammatory signaling while redox homeostasis remains unaffected

Systemic redox homeostasis is minimally perturbed by extended low pO_2 exposure

With our long-term objective being translation to humans, we studied the plasma proteome after investigating key molecular aspects of the lung proteome. However, before the proteome based investigation, we performed a basic biochemical investigation into redox parameters. Oxidative stress was estimated via ROS, TBARS and NOx levels (Fig. S4 a, b & c,*Supplementary information*). ROS and TBARS showed slight increase in HD3 with HD7 and HD14 tending towards normalized values of both. NOx, on the other hand, showed its minimal value in HD3 and maximal value in HD7 while HD14 had levels similar to Normoxia.

We then investigated central antioxidant molecules' concentration and activity in plasma (Fig. S4 d, e, f & g, *Supplementary information*). Contrary to our observations in previous study (Paul et al., 2018), we observe an anti-trend in TAC values in lung (Fig. S1d,*Supplementary information*) and plasma (Fig. S4d,*Supplementary information*). In HD3, TAC values show a decrease with sequential increases in both HD7 and HD14. HD14 had TAC value very similar to Normoxia TAC value. A similar trend is observed in catalase activity. SOD activity, on the other hand, remains constant across HD7 and HD14 after a slight increase in HD3. Total GSH also tends to remain constant throughout the low pO_2 exposed groups.

A hierarchical clustergram representing expression trends of redox-specific transcripts in blood of Normoxia, HD3, HD7 and HD14 group rats (Fig. S5, *Supplementary information*) revealed some redox responsive transcripts to have similar trends across HD3, HD7 and HD14 while some transcripts had similar expression in Normoxia, HD3, HD7 with opposing trends in Normoxia or HD14, respectively. Thus, non-directionality of the redox transcript-based assessment suggested redox stress homeostasis is maintained during extended exposure to low pO_2 8.19 kPa (observed at altitudes of 25,000 ft).

Global proteome analysis indicates STAT3 modulates lipid metabolism associated inflammatory signaling

Since redox stress management was ruled out as the main molecular event, we investigated the global plasma proteome using iTRAQ-LC-MS/MS (Table S2, *Supplementary information*) to ascertain the molecular events. In all groups, 312 proteins were identified and quantified. In HD3, 32 proteins were up-regulated and 42 were down-regulated. In HD7, 48 proteins were up-regulated and 36 proteins were down-regulated. In HD14, 60 proteins (the highest) were up-regulated and 31 proteins (the lowest) were down-regulated (Fig. 4a). This is in contrast to the lung proteome where across all groups, down-regulation of protein expression was dominant. The most significant molecular and cellular processes across groups were the same along with the same proteins involved in those processes (Fig. 4b). Cellular movement, Cell death and survival and Cell-to-cell signaling and interaction were also observed to be significant in the lung proteome. Protein synthesis and cellular compromise are unique to the plasma proteome. The trends of protein expression in the global proteome across HD3, HD7 and HD14 (relative to Normoxia) was pictured using hierarchical clustergram (Fig. 4c). The most significant canonical pathways were Acute phase response signaling (APRS), LXR/RXR Activation, FXR/RXR Activation, Coagulation system and Complement system (Fig. 4d). LXR/RXR remained down-regulated throughout the hypoxia exposure. Coagulation system remained up-regulated throughout

the environmental hypoxia exposure. Complement system is up-regulated sequentially in HD7 and HD14 after being neutral in HD3. FXR/RXR remains non-directional throughout the environmental hypoxia exposure. APRS, quite interestingly, is strongly up-regulated in HD3, gets down-regulated at HD7. In HD14, it becomes up-regulated again.

Since STAT3 is a regulatory protein for Acute phase response, we investigated the APRS with overlaid expression data from our plasma dataset (Fig. 5a). Furthermore, an integrated network created using the top five canonical pathways (Fig. 5b) to identify the upstream regulators. We observed an interplay of RXR with HNF4A, complement proteins, apolipoproteins and coagulation proteins. This was indicative of perturbations in lipid metabolism associated with perturbations in inflammatory signaling and coagulation cascades during extended exposures to low pO_2. To further verify these perturbations (in addition to their links with acute phase response) and assess antioxidant status we performed immunoblotting of proteins like glutathione peroxidase 3 (GPx3), plasminogen, C3, C4b, transthyretin (TTR), apolipoproteins A1 and H (Fig. 5c). GPx-3 was observed to be higher in HD3, similar to Normoxia in HD7 while HD14 showed lowest levels. C3 levels sequentially increased across the groups, reaching highest levels at HD14. C4b sequentially increased in HD3 and HD7, reaching its highest levels. In HD14, C4b levels were similar to HD3. Ttr levels decreased in HD3 and remained nearly constant in HD7 and HD14. Apo A1 levels were progressively and notably lower across HD3 to HD14 as compared to control group. Apo H levels were notably low throughout the environmental hypoxia exposure. Overall, upon increasing durations of low pO_2 exposures, particularly past day 7, there is increased inflammatory signaling, declining antioxidant reserves and adverse effects on lipid metabolism.

Indicators of cytoskeletal remodeling and inflammation in altitude acclimatized humans

The real-world manifestation of lowered pO_2 exposure for extended durations is observed during high altitude explorations. We observed that during extended exposure to low pO_2 in SD rats, lipid metabolism was favored while TCA cycle was subdued (Fig. 3c & d; RXR and MDH) indicating perturbed systemic energy homeostasis. However, results also indicate lowered HDL levels (incumbent on Apo A1 expression) as exposure of duration to lower pO_2increased (Fig. 5c). We found that it was associated with the lung cytoskeletal re-arrangements (Fig. 3d). The perturbed energy homeostasis resulted in innate immune activation, mostly inflammatory signaling, which was observed via acute phase response signaling (Fig. 5). Based on these aspects, we selected the following proteins that were specific for a signaling network/molecular process: alpha-1-antitrypsin, cofilin-1 and S100A8. These may not be called translated markers owing to the different time points at altitude as well as a different altitude zone as compared to the experimental conditions of the SD rats. The small sample size may also be considered a disqualification for the same. However, the results obtained (Fig. 6) do provide qualitative hints at the major processes that may be further analyzed for finding biomarkers of altitude acclimatization/disease and therapeutic targets in human samples.

Since innate immune activation and calcium associated proteins were observed in rat plasma (Table S1; Supplementary information), we measured S100A8 in human plasma (Fig. 6a). S100A8 inhibits neutrophil activation and adhesion and also modulates inflammatory processes (Raquil, Anceriz, Rouleau, & Tessier, 2008). It is a component of calprotectin (Yui, Nakatani, & Mikami, 2003), a fecal biomarker of gastrointestinal inflammation in irritable bowel syndrome (Van Rheenen, Van de Vijver, & Fidler, 2010). There were no major changes throughout HAD7, HAD30 and HAD120 as compared to Normoxia. This is indicative that innate immune processes, particularly inflammatory processes, are well-balanced in individuals acclimatized to low pO_2levels at high altitude. Next, we evaluated cofilin-1 levels in human plasma (Fig. 6b). Along with Lim kinase, cofilin signaling is essential for remodeling of cytoskeleton (Danen et al., 2005; Wang, Eddy, & Condeelis, 2007). Higher levels were observed in HAD7 suggesting increased cytoskeletal re-modeling corroborated by increased redox stress in HD3 in rats. Its near Normoxic levels in HAD30 and HAD120 suggest a balanced impetus for actin dynamics. However, till day 7 cofilin-1 levels are higher than control group. To delve further into this aspect, alpha-1-antitrypsin (Tables S1 and S2; Supplementary information) was also assayed in human plasma (Fig. 6c). It was observed in the Acute phase response signaling in SD rats. It is known to inhibit elastase and thus prevents lung tissue damage during various conditions (Brantly et al.,

1988; Garratt et al., 2016; Gøtzsche & Johansen, 2016). In a previous study, we had shown that Acute phase response signaling is a homologous pathway between rat and human exposed to hypoxia (Paul, Bhargava, & Ahmad, 2017). Alpha-1-antitrypsin levels were investigated in human plasma based on our findings that cytoskeletal re-arrangement was taking place in the rat lung. We observed that plasma alpha-1-antitrypsin levels are lowered throughout the high altitude exposure in humans till HAD30 suggesting increased chances of lung tissue damage/re-modeling. However, it's increased levels at HAD120 suggest better adjusted lung cytoskeletal dynamics in agreement with recovering cofilin-1 levels.

Discussion

In a preceding study, we observed redox homeostasis to be the single causal event that determined the overall response of both lung and plasma proteome to acute low pO_2 exposure (Paul et al., 2018). In the case of extended environmental hypoxia exposure, redox homeostasis was not the defining impetus, after day 3. Our initial investigations into biochemical redox parameters in both lung and plasma samples revealed ROS and TBARS to be near normoxic values in both HD7 and HD14. Even NOx levels confirmed to Normoxic levels in HD14 lung tissues and plasma. The overall antioxidant defenses in both lung and plasma were adept at disposing off excess radicals. TAC trends were opposing in lung and plasma. While lung showed the highest TAC values in HD3 with subsequent lowering till HD14, plasma had lowest TAC levels in HD3 with subsequent increases till HD14. Plasma had nearly similar total GSH concentrations across groups. However, lung tissue saw increased GSH concentrations in HD3 and HD7 with normalized GSH concentrations in HD14. SOD and catalase levels in both lungs and plasma had similar activity levels across all experimental groups relative to cata. We also failed to observe any redox stress/homeostasis mediated differential trends in the redox specific PCR array in both lung and plasma of the experimental groups (Fig. S1 and S3,*Supplementary information*). Thus, after 3 days of exposure to environmental hypoxia, we may conclude that a systemic redox homeostasis, at least at the level of proteome, is achieved. Sudden lowering of pO_2 (observed during rapid ascent to altitude) is a causal factor of acute altitude illnesses (Barry & Pollard, 2003; Fiore, Hall, & Shoja, 2010) and redox stress being an underlying molecular event of acute altitude illnesses (Damian Miles Bailey, Bärtsch, Knauth, & Baumgartner, 2009; Damian M Bailey & Davies, 2001), slow graded lowering of pO_2 in this study may be aiding redox homeostasis. Probably due to the slower ascent rates (250 m/min), even in presence of redox stress (noticeably till HD3), longer hypoxic durations enabled the organism to attenuate its effects. Such an effect is the uneven expression of housekeeping proteins which was observed in a previous study concerning rapid ascent to altitude (Paul et al., 2018). In this study, abrogation of such effects was observed in GAPDH expression profile (Fig. 3c & d). Given the susceptibility of housekeeping proteins' expression to rapid extreme hypoxia exposure, they are pivotal in deciphering whether an organism is adapting to hypoxia.

Further exploration of the lung proteome revealed that most of the differentially expressed proteins were involved in cytoskeletal re-arrangements. The major canonical pathways were Remodeling of epithelial adherens junctions, Actin cytoskeleton signaling and Signaling by Rho-family GTPases. As most of the differentially expressed lung proteins were down-regulated, especially in HD3, we observed the directional canonical pathways to be either neutral (Remodeling of epithelial adherens junctions) or down-regulated (Actin cytoskeleton signaling, Signaling by Rho-family GTPases). Although Clathrin mediated endocytosis signaling and Epithelial adherens junction signaling did not show any trends, their presence among the top canonical pathways implicated lipid derived processes and internalization of protein channels as two features to be further investigated. Further since both clathrin coated pits and adherens junctions are cell surface features, extended low pO_2 exposure is thus managed by modulation of lung cell surface features. Adherens junction formation is closely linked to biochemical, mechanical and cytoskeletal events, particularly Actin cytoskeleton.

The overall downregulation pathways like Actin cytoskeleton signaling points to a bias towards internalization of clathrin coated pits and lowered turnover of adherens junction assembly/disassembly in order to maintain homeostasis considering the lowered energy status of hypoxic tissues/cells. Actin cytoskeleton signaling is mainly involved in modulation of cell shape and external structures in response to external stimuli (Schmidt

& Hall, 1998). Actin cytoskeleton signaling has been previously reported to be associated with integrins (DeMali, Wennerberg, & Burridge, 2003; Wu & Dedhar, 2001), Rho-family (Maekawa et al., 1999; Winter et al., 2001) and even T-cell activation (Dustin & Cooper, 2000; Valitutti, Dessing, Aktories, Gallati, & Lanzavecchia, 1995). Integrin based signaling is also involved in modulation of signaling by Rho-family GTPases, another significantly up-regulated canonical pathway in the lung, via Rac and Cdc42 (DeMali et al., 2003). Further Actin cytoskeleton signaling via Integrin beta 1 may also affect the phosphorylation trends through the action of Tyrosine kinases (Kornberg, Earp, Turner, Prockop, & Juliano, 1991). The interplay between Rho-GTPase family, actin cytoskeleton and epithelial adherens junction remodeling suggests increased cell movement, formation of cell-cell junctions and may indicate structural changes in the lungs to withstand the lower ambient partial pressure. Stress failure of pulmonary vasculature is implicated in development of HAPE (Stream & Grissom, 2008; West et al., 1995; West & Mathieu-Costello, 1992). Since these rats can be considered acclimatized in the absence of any mortality or signs of altitude illnesses, the above canonical pathways can be speculated to modify the lung cellular structures in such a manner as to prevent stress failure in alveolar capillaries. As in the previous study (Paul et al., 2018), STAT3 was observed to be an upstream regulator present in lung tissue and affecting the global proteome. RXR was also observed to show differential signaling.

In the plasma proteome, we observed APRS signaling and LXR/RXR Activation as the two most significant canonical pathways. STAT3 is an up-stream regulator of APRS while RXR is involved in LXR/RXR Activation pathway. In the plasma, STAT3 via APRS triggered perturbations in multiple apolipoproteins (also downstream of LXR/RXR mediated signaling), complement proteins (components of Complement system) and proteins associated with coagulation cascades. APRS is activated in HD3 and HD14 while its down-regulated in HD7. This could be due to the lesser degree of activation of Coagulation system in HD7. The consistently lower levels of Protein C and Protein S during extended low pO_2 exposure (Fig. S6, *Supplementary information*) indicate increased clotting tendency. This is corroborated by previous reports suggesting exaggerated occurrence of thrombosis during extended low pO_2 exposure at high altitude involving low levels of Protein C & protein S (Anand, Jha, Saha, Sharma, & Adya, 2001; Boulos, Kouroukis, & Blake, 1999; Fujimaki, Matsutani, Asai, Kohno, & Koike, 1986; Le Roux, Larmignat, Marchal, & Richalet, 1992; Nair et al., 2008). Complement system shows sequential up-regulation across groups with HD14 showing highest degree of up-regulation (Fig. S7). However, across the key complement proteins like C3 and C4b (Fig. 5c), we observed a very slight increase in HD14 suggesting minimal chances of a strong complement cascade. In our analysis, we observed that APRS signaling proteins provide the most comprehensive clues regarding survival of rats at low pO_2. We observed antioxidants (thioredoxin, glutathione peroxidase); transporters (transthyretin); metabolic proteins (Apo A1) and inflammatory proteins (C3) to be indicative of survival at low pO_2 for SD rats.

Based on the processes that were observed to be perturbed in the rats exposed to extended durations of low pO_2, we evaluated particular proteins known to be associated with these processes in humans exposed to chronic durations at altitude. These proteins were essentially qualitative indicators for the specific process. We observed that acute phase signaling was observed to be perturbed. Thus, we assessed alpha-1-antitrypsin in human plasma. It serves as a connector between acute phase response and cytoskeletal remodeling cascades. We observed cytoskeletal re-modeling in rat and assessed cofilin-1 in human plasma. Similarly, S100A8 was assessed in human plasma as innate immune activation was observed in rat. Each of the above three proteins that were measured using ELISA were picked from the LC-MS/MS dataset (*Tables S1 & S2, Supplementary information*) owing to their crucial roles in the molecular processes that were perturbed by extended exposure to low pO_2 during high altitude stay.

Limitations

An important limitation of this study was the logistical limitation in exposing the animals to even longer low pO_2 durations lasting 30 or more days. Another minor limitation of this study was the inability to directly juxtapose the lung proteome and plasma proteome owing to technical limitations in IPA. Both had to be analyzed separately.

Human volunteers recruited for the study, although picked at random from a pool of eligible volunteers, still represent a very small sample size for any statistical robustness of the plasma proteins analyzed. These proteins are only qualitative indicators for the processes mentioned and require larger cohort based studies in the future.

Conclusion

This study provided insight into the changes in molecular events during the transition from acute to extended low pO_2 exposure in the lung and plasma proteome. The molecular events which respond to extended low pO_2 exposure are Cytoskeletal re-modeling, energy metabolism, redox homeostasis and innate immunity/inflammation (Fig. 7). STAT3 was observed to play a connecting role between the molecular occurrences in the lung and the systemic proteomic response during extended low pO_2 exposure. We identified three pathways that can provide biomarkers and therapeutic targets against extended low pO_2 exposures at altitude. These are cytoskeletal elements, energy metabolism and inflammation. Normalized redox homeostasis, even at the cost of perturbed energy metabolism and associated inflammation, was key to survival over longer low pO_2 exposure time periods. We also provided indicators in human plasma (alpha-1-antitrypsin, cofilin-1 and S100A8) for cytoskeletal re-arrangements and inflammation. It was observed that innate immune/inflammatory processes were balanced in individuals successfully surviving in low pO_2 for extended durations.

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Conflict of interest

Authors declare no conflict of interest.

Author contribution

YA conceptualized the manuscript and supervised data analysis. AG and SP performed the experiments and analyzed the data. SP and AG wrote the manuscript. AA conceptualized the figures according to the data and provided scientific inputs during manuscript preparation. YA and KB critically evaluated the manuscript.

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Figures and figure legends

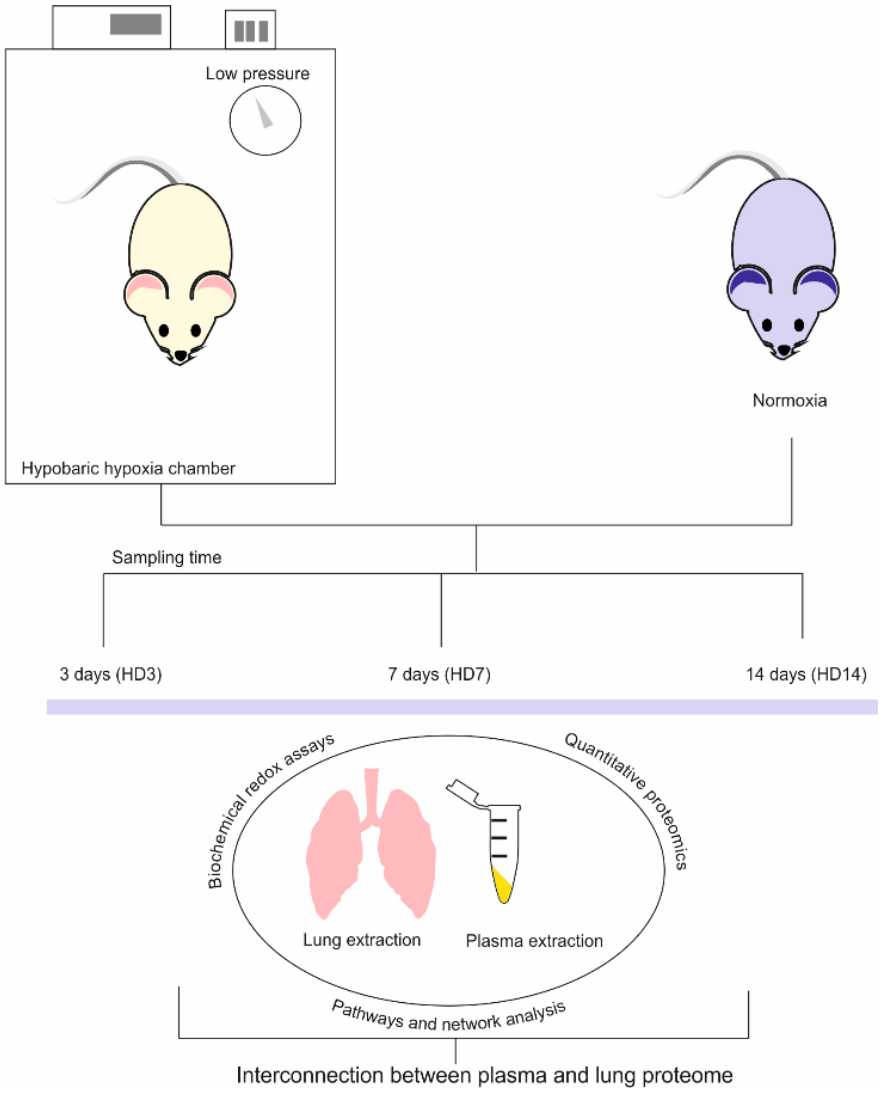


Fig. 1. Study design. Male SD rats (8-12 weeks; 230-240 g) were exposed to hypobaric hypoxia corresponding to P_B 40 kPa for 3 days, 7 days and 14 days. Immediately after the exposure the animals were sacrificed. The lungs and plasma were collected for subsequent biochemical redox assays, quantitative proteomics and network analysis.

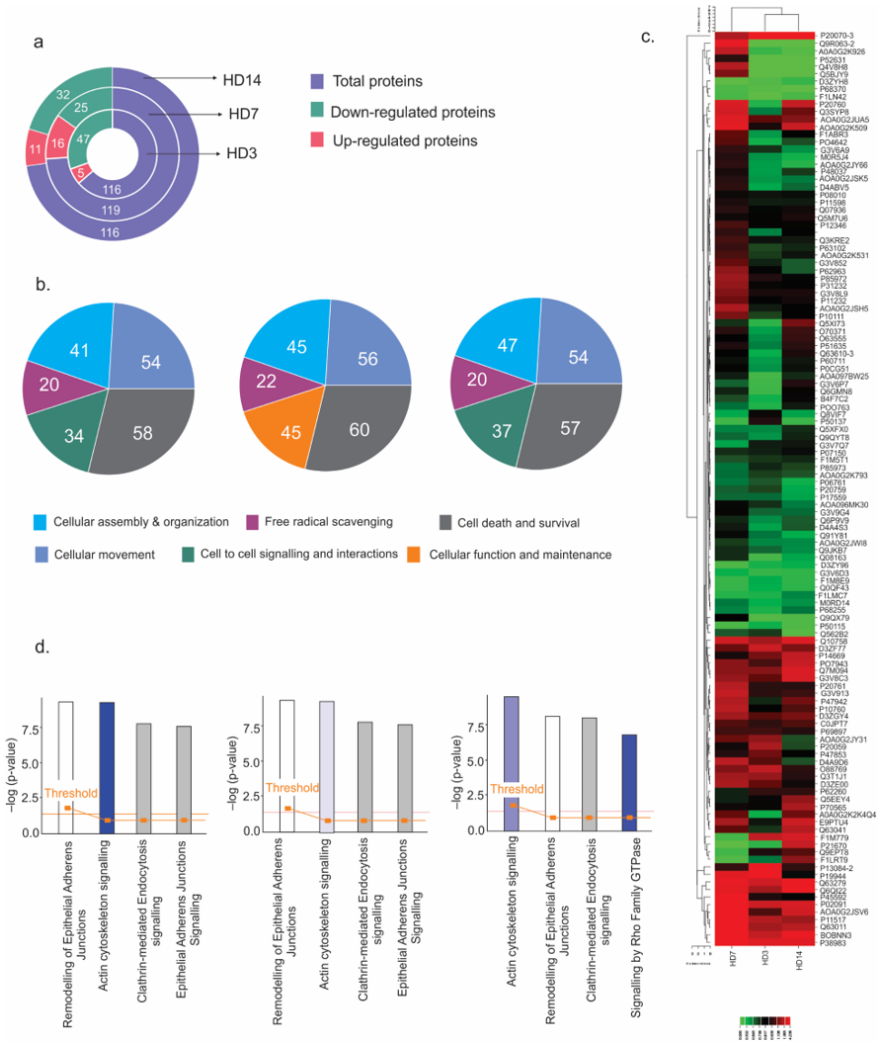
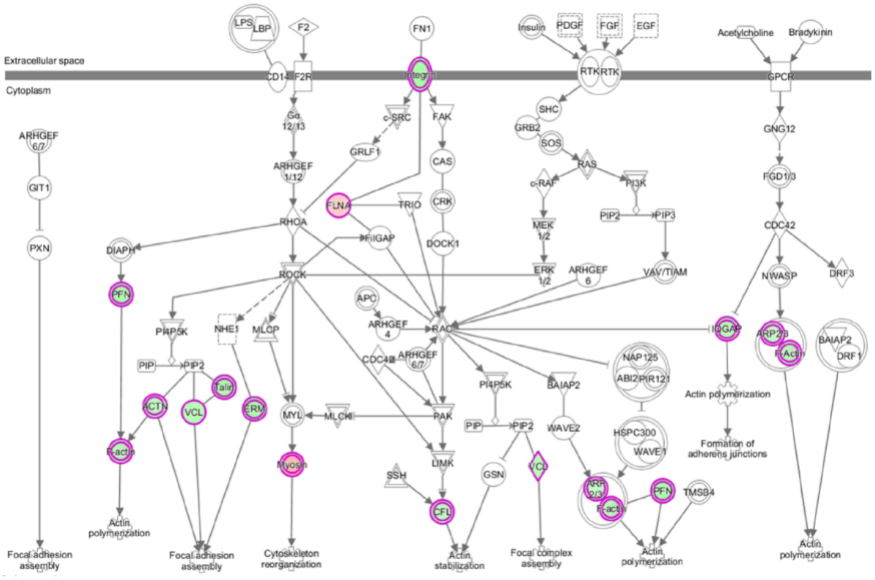
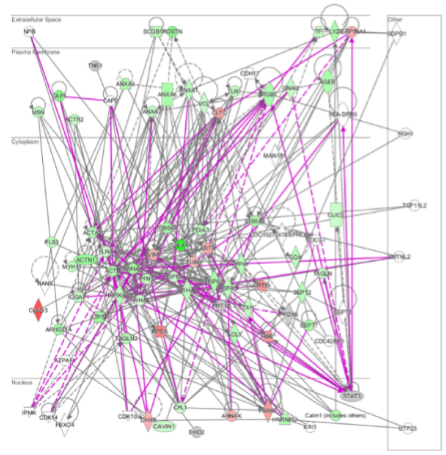
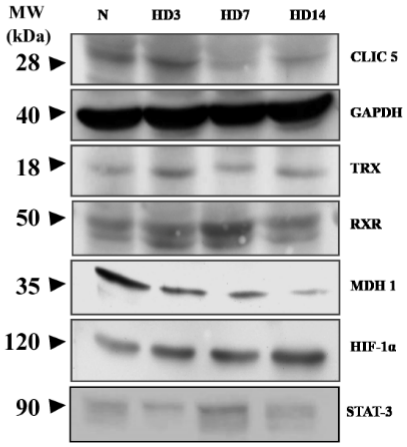


Fig. 2. a. Overview of the identified differentially expressed lung proteins. **b.** Pie chart showing the most significant molecular and cellular processes across each hypoxic group using normalized protein fold change values. **c** . Hierarchical clustergram of identified lung proteins using normalized fold change values. Green is down-regulated and red is up-regulated **d** . IPA based analysis of the most significant pathways as well their overall directionality based on z-scores (blue: down-regulation; white: neutral; grey: no directionality).







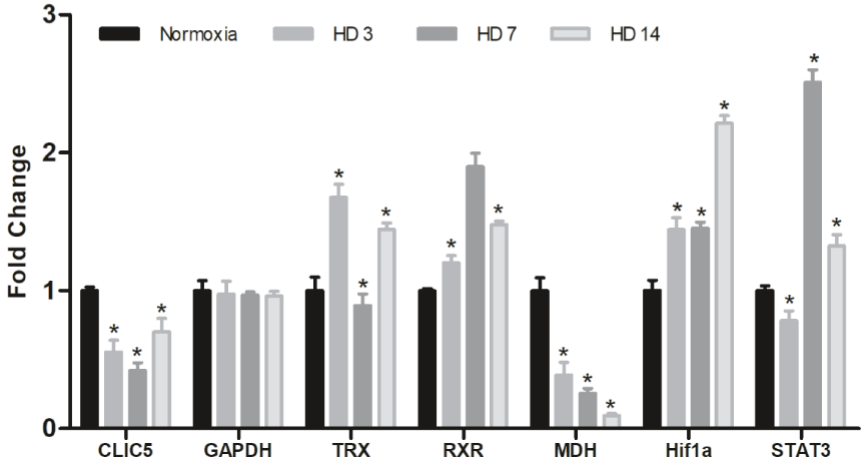


Fig. 3. a. Actin cytoskeleton signaling pathway. IPA mined Actin cytoskeleton network overlaid with protein expression data from the lung protein dataset. Green signifies down-regulation and pink/red signifies up-regulation of proteins. **b. Role of STAT3 in modulation of energy metabolism, structural proteins and antioxidant proteins.** IPA based integrated network for HD3, HD7 and HD14 datasets (using lung proteome expression data) showed STAT3 as the upstream regulator. **c.** Representative immunoblots of key lung proteins along with equal loading gel (Fig. S6: loading control; Supplementary information) and **d.** their bar-graphs. Results presented as Mean±SEM. *p-value<0.05.

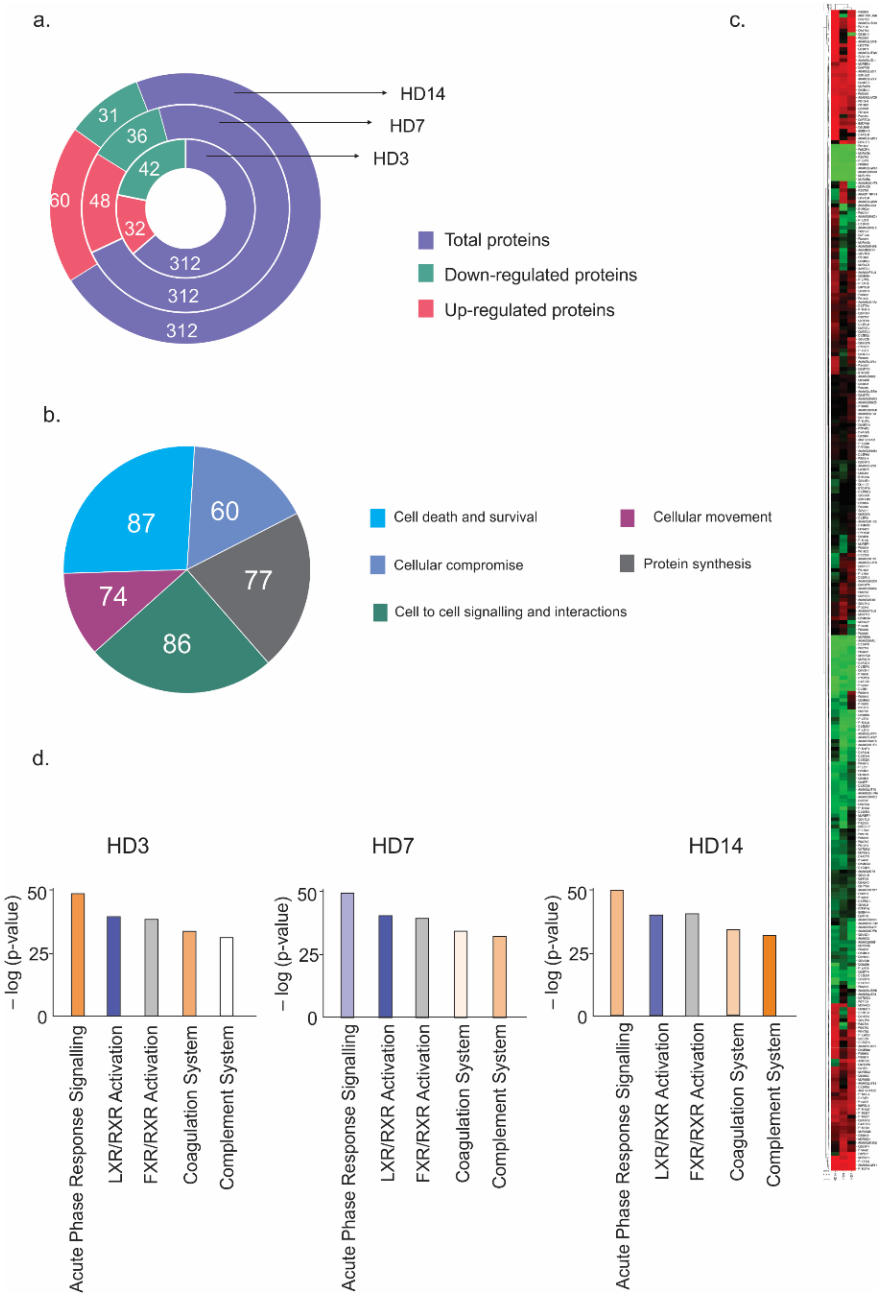


Fig. 4. a. Overview of the identified differentially expressed SD rat plasma proteins. **b.** Pie chart showing the most significant molecular and cellular processes across hypoxic groups using normalized protein fold

change values. **c.** Hierarchical clustergram of identified plasma proteins using normalized fold change values. Green is down-regulated and red is up-regulated **d.** IPA based analysis of the most significant pathways as well their overall directionality based on z-scores (orange: up-regulation; blue: down-regulation; white: neutral; grey: no directionality).

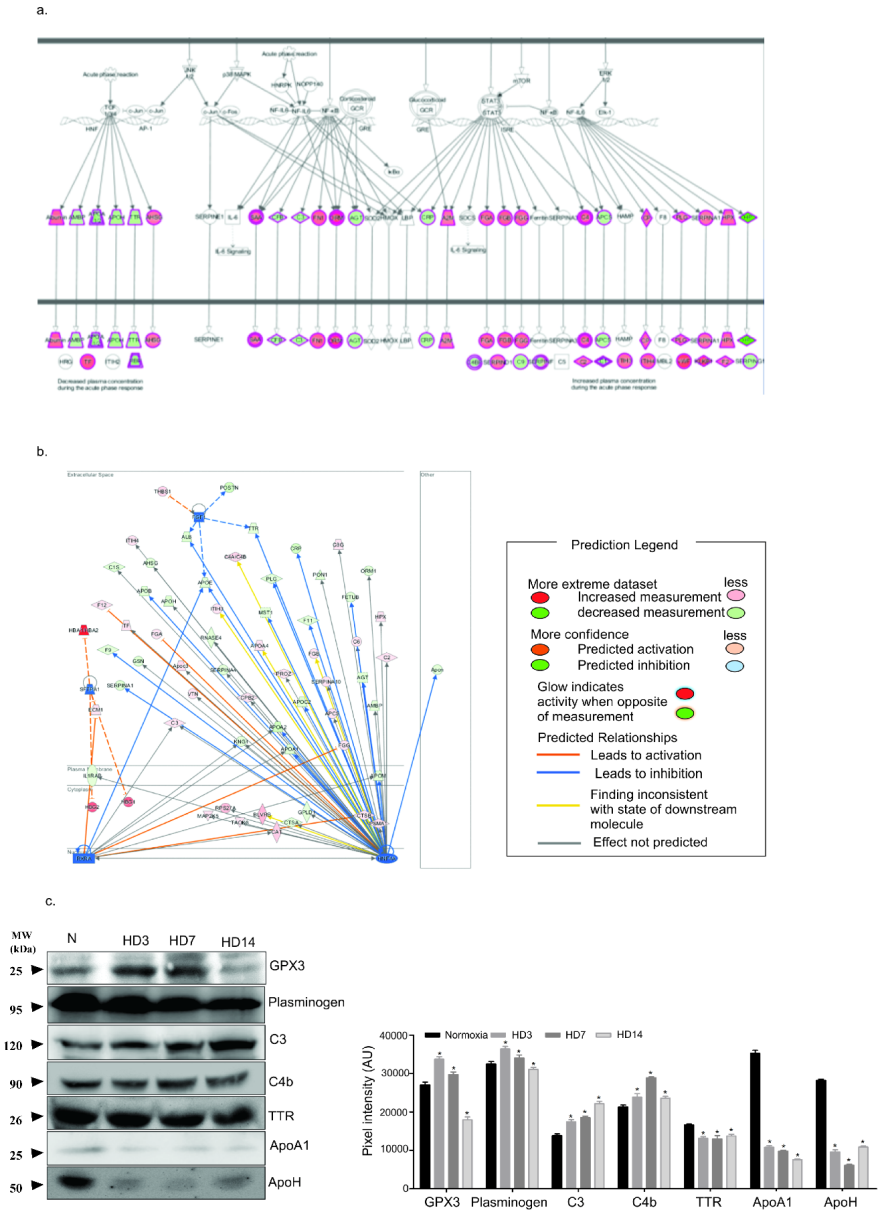


Fig. 5. Acute phase response signaling. a. IPA mined acute phase response network overlaid with protein expression data from the plasma protein dataset. Green signifies down-regulation and pink/red signifies up-regulation of proteins**. b. Interplay between lipid metabolism, inflammation and coagulation system.** Upstream regulators identified across groups using IPA. RXR is observed to interact with HNF4A, apolipoproteins, coagulation proteins, cytoskeletal proteins and complement proteins thus suggesting interplay of lipid metabolism, cytoskeleton, coagulation system and inflammation. **c.**Immunoblots of relevant acute phase proteins involved in lipid metabolism, complement system, coagulation system and

redox homeostasis. Results represented as Mean±SEM. *p-value<0.05.

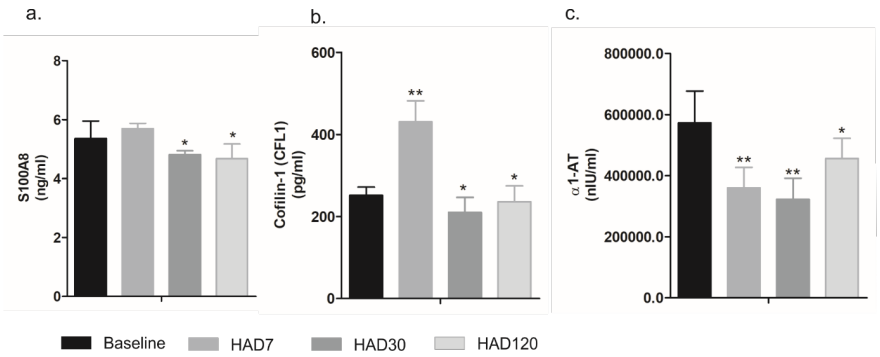


Fig. 6. Process specific proteins in humans undergoing extended low pO_2 exposure. ELISA results for**a.** S100A8, **b.** Cofilin 1 (CFL 1) and **c.**alpha-1-antitrypsin (α-1-AT) in human plasma samples after 7, 30 and 120 days at 14,000 ft (pO_2 12.81 kPa) represented as Mean±SEM. *p-value<0.05. **p-value<0.01. Across rat model and humans exposed to low pO_2 for extended durations, qualitative similarities exist as shown by perturbed inflammation cascades (S100A8) and cyto-skeletal re-arrangements (CFL 1 and α-1-AT).

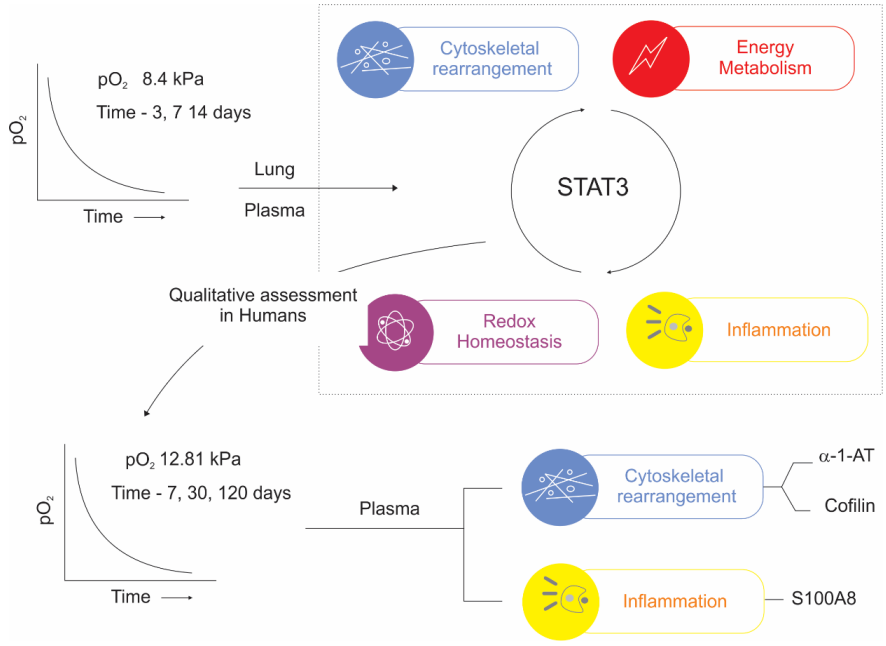


Fig. 7. Extended durations of low pO_2 exposure caused cytoskeletal re-arrangements, perturbations in energy metabolism and inflammatory signaling in order to maintain redox homeostasis. STAT3 was a central protein modulating these processes. We qualitatively assessed cytoskeletal re-arrangements and inflammation by assaying specific proteins (using ELISA) in human plasma samples that were obtained from human volunteers undergoing extended high altitude stay (low pO_2 conditions). Cytoskeletal re-arrangements (cofilin and α-1-AT) and inflammation (S100A8) were observed to be perturbed in humans.