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ORIGINAL ARTICLE

Quantitative proteomics reveal an altered pattern of protein expression in saliva of hypobaric hypoxia-induced rat model

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Abstract

Hypobaric hypoxia (HH), a pathophysiological condition caused by an ascent to a high altitude. It occurs due to a deficiency of oxygen at the tissue level. Saliva is an advantageous biological sample because of easy, low cost and minimally invasive sample collection and processing. Although few reports have documented the effect of long term exposure to hypobaric hypoxia on salivary proteome but short term proteome based study remains uninvestigated. In this study, age-matched male SD rats were exposed to simulated hypobaric hypoxia (~25,000 ft, 7620 m) for 1 day (HD1), 3 days (HD3) and 7 days (HD7) followed by iTRAQ based LCMS/MS comparison with normoxic controls. Oxidative stress being the main event of hypobaric hypoxia suggested an initial increase in levels of oxidative stress parameters such as reactive oxygen species (ROS) and lipid peroxidation; and decrease in antioxidant enzymes such as the activity of catalase and reduced glutathione. Based on iTRAQ, out of 67 differentially expressed proteins, 36 (22 up-regulated and 14 down-regulated), 45 (24 up-regulated and 21 down-regulated), and 45 (19 up-regulated and 26 down-regulated) were modulated in HD1, HD3, and HD7 group respectively. Few proteins such as BPI fold-containing family A member 2, cystatin, and carbonic anhydrase 1 showed many folds differential expression in exposed groups as compared to normoxia. Through Ingenuity Pathways analysis, we have observed glucocorticoid receptor signaling and MSP-RON pathway to be most significantly affected. Glucocorticoid receptor signaling and MSP-RON signalling being the most significant pathways involved modulated proteins such as annexin 1, hsp90, keratin, actin-beta, and kallikrein 1. These proteins were found to aid in acclimatization and survival during hypobaric hypoxia. Taken together, this study provided the proof of concept for footprints of hypobaric hypoxia-mediated events during short term exposure through rat salivary proteome.

Keywords Rat saliva · Hypobaric hypoxia · Proteomics

Abbreviations

iTRAQ	Isobaric tags for relative and absolute quantification
LCMS/MS	Liquid chromatography based mass spectrometry
HH	Hypobaric hypoxia

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Introduction

Hypobaric hypoxia (HH) is characterized by deprived oxygen at tissue level due to the reduced atmospheric partial pressure of oxygen. Multiple clinical manifestations, such as shortness of breath, headache, dizziness, tachycardia, acute mountain sickness (AMS), pulmonary and cerebral edema (HAPE and HACE), are caused by oxidative stress related to hypobaric hypoxia (Hackett and Roach 2004; San et al. 2013; Sharp and Bernaudin 2004; Wilson et al. 2009). Various researchers have suggested the usage of SD rats for understanding human patho-physiologies and diseases (Gumprecht et al. 1993; Wu et al. 2004; Reaven 1991; Cai et al. 2006; Jin et al. 2008; Ramos et al. 2003; Patlolla et al. 2009) and hypobaric hypoxia using different biological samples such as plasma, serum, and tissues (Ma et al. 2015; Ou and Smith 1983; Underwood et al. 1998; Yang et al. 2012; Yuan et al. 2008; Kumar et al. 2014;

Anamika Gangwar et al. 2019). Also, rat and human share common protein networks in relation to hypobaric hypoxia as suggested in recent meta-analytical evidence (Paul et al. 2017a). Thus, the SD rat model was selected for studying the effects of hypobaric hypoxia.

Saliva being a source of broad-spectrum of biomolecules (mainly proteins, lipids, hormones, and nucleic acids that originated from various local/systemic sources) holds promising future among diagnostic samples (Hu et al. 2006; Denny et al. 2008). It is a clear slightly acidic biological fluid secreted from major (parotid, submandibular and sublingual glands) and minor salivary glands (labial, buccal, lingual, and palatal glands) (Yoshizawa et al. 2013; Malathi et al. 2016; Lamy et al. 2010). Saliva is an advantageous biological sample because of easy, low cost and minimally invasive sample collection and processing (Yoshizawa et al. 2013; Campo et al. 2006; Chiappin et al. 2007; Kaczor-Urbanowicz et al. 2017; Schafer et al. 2014). In the context of saliva as a biological sample, in vivo model has been used for understanding modulations behind various non-pathological conditions such as psychosocial stress and pathophysiological conditions such as periodontal disease, Sjogren's syndrome, diabetes, Chagas disease, etc. (Nayar et al. 2016; Alves et al. 1994; Colaianna et al. 2013; Martinez et al. 1979; Nakamura-Kiyama et al. 2014). In terms of hypobaric hypoxia, previous reports suggested decreased secretory capacity and histological structure of rat salivary glands under the effect of chronic hypoxia (Elverdin et al. 1995; Scott and Gradwell 1989). Also, a study suggested three months of chronic continuous hypoxia exposure to rats resulted in lower gum density, alveolar bone loss, and decreased antioxidant capacity that indicated a deleterious effect on oral health (Terrizzi et al. 2016).

Due to inadequate research on rat saliva proteome during short term exposure to hypobaric hypoxia, it was encouraged to analyze salivary proteome in response to hypobaric hypoxia through proteomics approach in an in vivo model for identifying multiple molecular signatures involved in different systemic processes. In this study, age-matched male SD rats were divided into four groups such as normoxic controls (N) and rats exposed to hypobaric hypoxia (~ 25,000 ft, 7620 m) for a short duration of 1 day (HD1), 3 days (HD3) and 7 days (HD7). After exposure, saliva was collected from the experimental groups and biochemical parameters were performed. Following this, the saliva samples from each experimental group were labeled with iTRAQ reagent and processed for LC–MS/MS. Then, differentially expressed proteins in the exposed groups as compared to normoxic controls were subjected to IPA software to identify relevant canonical pathways.

Materials and methods

All the chemicals unless specified were obtained from Sigma-Aldrich, USA.

Collection of whole rat saliva

Experimental animals used were age-matched male Sprague–Dawley rats weighing 200–230 g housed in polypropylene cages with paddy husk as substratum at 25 ± 5 °C with humidity at 50 ± 5% and a day-night cycle of 12 h each. Approval for animal procedures and experimental protocols were affirmed by Institutional Animal Ethics Committee (Authorization Number: 27/1999/CPCSEA) according to the standards set forth in the Guide for the Care and Use of Laboratory Animals (National Academy of Science, Washington, D.C.).

Twenty-four rats ($n = 24$) were randomly divided into four groups of six rats each, first normoxic controls (animals without simulated hypobaric hypoxia exposure; 500 ft), HD1 (animals exposed to simulated hypobaric hypoxia equivalent to 25,000 ft for 1 day), HD3 (animals exposed to simulated hypobaric hypoxia equivalent to 25,000 ft for 3 days) and HD7 (animals exposed to simulated hypobaric hypoxia equivalent to 25,000 ft for 7 days). Animals received a pre-exposure at 15,000 ft for 10 h followed by 1 h exposure at 500 ft prior to exposure of 25,000 ft for 24 h (Paul et al. 2017b).

Hypobaric hypoxia exposure and sample collection

Hypobaric Hypoxia exposure simulation was performed in custom-designed hypobaric hypoxia simulation chamber (7 star systems, Delhi, India) with a constant temperature and humidity of 25 ± 5 °C and 50 ± 5%, respectively and an ascent rate of 589 m/min during the exposure. The airflow of 2 L/min was maintained in the chamber and food and water was provided to the animals inside the chamber. Immediately after completion of the exposure, individual rat saliva secretion was induced with an intraperitoneal injection of pilocarpine and collected by micropipette from the mouth followed by centrifugation at 16,000×*g* for 5 min at 4 °C to remove particulate matter (Lamy et al. 2010; Costa et al. 2008). Protease inhibitor cocktail (Cat # P8340, Sigma, USA) was added to prevent protease activity. All the samples were stored in -80^{0}C until further use.

Estimation of oxidative stress parameters

Reactive oxygen species (ROS) estimation

In saliva, estimation of Reactive Oxygen Species (ROS) levels was performed using a fluorescent dye 2′,

7′-Dichlorofluorescin diacetate (DCFDA) (Cat. No. D6883). In the presence of intracellular reactive oxygen species, the dye enters through the cell membrane and cleaves into 2, 7-dichlorofluorescein by the action of intracellular esterase enzymes and produces fluorescence. The generated fluorescence is directly proportional to the reactive oxygen species levels (Wang and Joseph 1999). In brief, 150 µl of undiluted saliva along with 5 µl of 2 mM Dichlorofluorescin diacetate was incubated for 40 min at room temperature in amber tubes in dark. Following incubation, 2 ml of PBS was added to the tubes and 200 µl of each sample was added to 96-well plate (PBS as blank). Fluorescence was measured at excitation and emission wavelengths of 485 nm and 531 nm respectively using an ELISA plate reader (Synergy H1 microplate reader, BioTek USA).

Lipid peroxidation estimation using thiobarbituric acid reactive substances (TBARS) estimation

Thiobarbituric acid reactive substances (TBARS) assay was performed to estimate lipid peroxidation in saliva. It was measured directly using QuantiChrom™ TBARS Assay Kit (Cat. No. DTBA-100, Bioassays Systems, CA, USA) as suggested by the manufacturer's protocol. In short, standards and samples were prepared by diluting with milli-Q and precipitating with ice-cold 10% TCA respectively. Following this, TBA was added to the diluted standards and saliva supernatant and incubated at 100 ℃ for 1 h in a water bath. The mixtures were then allowed to cool to RT. Afterwards, 200 µl of the mixtures were poured onto 96-well plate (TBA as blank) and the absorbance was measured at 535 nm using a spectrophotometer (EON Biotech, USA).

Measurement of antioxidant status

Estimation of reduced glutathione

In saliva samples, levels of reduced glutathione were measured by microplate assay kit (Cat. No. CS0260) as suggested by the manufacturer. In short, standards were reconstituted and serially diluted with milli-Q and 5% 5-sulfosalicylic acid (SSA) respectively. Standards and samples along with working mixture (assay buffer, enzyme and 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) solution) were incubated for 5 min at RT followed by addition of NADPH solution to each well. Kinetic, as well as end-point absorbance, were recorded starting from 0 to 5 min (1 min interval) at 412 nm using an ELISA plate reader (EON Biotech, USA).

Estimation of catalase activity

In saliva, the activity of catalase enzyme was estimated using EnzyChromTM Catalase Assay Kit (Cat No. ECAT-100, Bioassay systems, USA) according to the manufacturer's instructions. In brief, 4.8 mM H_2O_2 was used to prepare standards. 10 µl of the standards or samples along with 90 µl of 50 µM H_2O_2 substrate were added onto the microplate and incubated for 30 min at RT. Further, the plate was incubated with 100 µl of detection reagent for 10 min at RT and the absorbance was recorded at 570 nm using a microplate reader (EON Biotech, USA).

Measurement of nitric oxide (NO) metabolites

Measurement of nitric oxide (NO) metabolites such as nitrate and nitrite was performed in saliva samples by a fluorometric assay using Oxiselect nitric oxide assay Kit (Cat No. STA-801, Cell Biolabs, CA, USA) according to manufacturer's instructions. In short, standards were diluted with milliq and samples were filtered using zeba spin desalting columns (Cat No. 89890, Thermo Fischer Scientific, USA) as per the manufacturer's protocol prior to NO estimation. 50 µl of standards or samples along with 50 µl of reaction mixture containing assay buffer and enzyme cofactor was added onto the microtiter plate and incubated for 1 h at RT on a rocking platform in dark. 20 µl of DAN fluorometric probe was then added to the wells and the plate was incubated for 10 min followed by incubation with 10 µl of stop solution for another 10 min. Fluorescence was recorded at excitation and emission wavelengths of 362 nm and 440 nm respectively using a spectrophotometer (Synergy H1 microplate reader, BioTek USA).

High throughput proteomics using LC–MS/MS

Sample preparation

100 µg of protein from saliva samples ($n = 6$ from each group) was dispensed in 30 µl of dissolution buffer and processed for LC–MS/MS using iTRAQ kit (Cat No. 4352135, ABSciex, USA). Processing includes three steps: (a) denaturation using 1 µl of denaturant, (b) reduction by incubation with 2 µl of reducing agent at 60 °C for 1 h and (c) alkylation using 1 µl of cysteine blocking agent followed by overnight in-solution trypsin digestion using trypsin singles proteomics grade (Cat no. T7575). The digested peptides were then labeled with iTRAQ reagents (activated with isopropanol) and incubated for 2 h at RT. SCX cartridge (5 micron, 300 Å bead from ABSciex, USA) was used to fractionate samples into fractions eluted at 35 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 250 mM, 350 mM, 500 mM ammonium formate buffer (ammonium formate, 30% v/v ACN and 0.1% formic acid; pH = 2.9) and got analyzed individually on quadrupole-TOF hybrid mass (Triple TOF 5600 & 6600, Sciex USA) spectrometer coupled to an EksigentNanoLC-Ultra 2D plus system.

Mass spectrometry (LC–MS/MS)

Each sample fraction was poured onto a trap column (200 µm × 0.5 mm) and desalted at flow rate 2 µl/min for 45 min followed by peptides separation using a nano-C18 column (75 µm × 15 cm) through a gradient method with buffer A (99.9% LC–MS water + 0.1% formic acid) and buffer B (99.9% acetonitrile + 0.1% formic Acid). Data were obtained in an Information dependent acquisition mode with MS settings such as nebulizing gas of 25; a curtain gas of 25; an Ion spray voltage of 2400 V and heater interface temperature of 130 °C followed by TOFMS scan in the mass range of 400–1600 m/z with an accumulation time of 250 ms and, the MS/MS product ion scan in the mass range of 100–1800 m/z for 70 ms with a total cycle time of 2.05 s approximately. Parent ions with abundance > 150 cps and with a charge state of + 2 to + 5 were selected for MS/MS fragmentation followed by exclusion of its mass and isotopes for 3 s. Further, MS/MS spectra were obtained with an adjust collision energy setting with high sensitivity mode when using iTRAQ reagent. Abundance Ratios > 1.5 were classified as up-regulated, < 0.67 were classified as down-regulated. Ratios from 1.5–0.67 were considered moderate to no changes. The experiment was performed in triplicates.

Pathway analysis of proteomics data

Results obtained from high throughput LC–MS/MS were sorted based on FDR < 1 (false discovery rate) and p value < 0.05. Selected proteins with their respective abundance ratios were analyzed using a network analysis tool, Ingenuity Pathway analysis (IPA, Qiagen) with an inbuilt statistical analysis package. Fold change ≥ 1.5 was set as a cutoff for various programs such as canonical pathways, disease networks and functions, and protein networks. These networks played an essential role in suggesting key changes in cellular events, biochemical processes, and molecular cascades. To predict the directionality of the cellular event, a significant positive and negative z-score was used. Top canonical pathways and molecular events were selected using minimum p value criteria.

Results

Redox signaling including oxidative parameters and antioxidant status

The main event of hypobaric hypoxia is oxidative stress, thus, we analyzed the levels of oxidative stress parameters. In oxidative stress parameters, the levels of reactive oxygen species (ROS) and lipid peroxidation were measured in rat saliva samples. The levels of ROS were found to be increased in the HD1 group followed by a decrease in HD7 group as compared to normoxic controls (Fig. 1a). The lipid peroxidation levels were found to be normalized in HD1 group, increased in HD3 group followed by a slight decrease in HD7 group as compared to normoxic controls (Fig. 1b).

Antioxidants provide protection to the damage caused by oxidative stress, thus, various antioxidants such as the activity of catalase and reduced glutathione were observed in rat saliva. Catalase activity was not found to be modulated in the HD1 group followed by an increase from HD3 group to HD7 group as compared to normoxic controls (Fig. 2a). The levels of reduced glutathione also showed different patterns such as the levels were decreased from normoxic

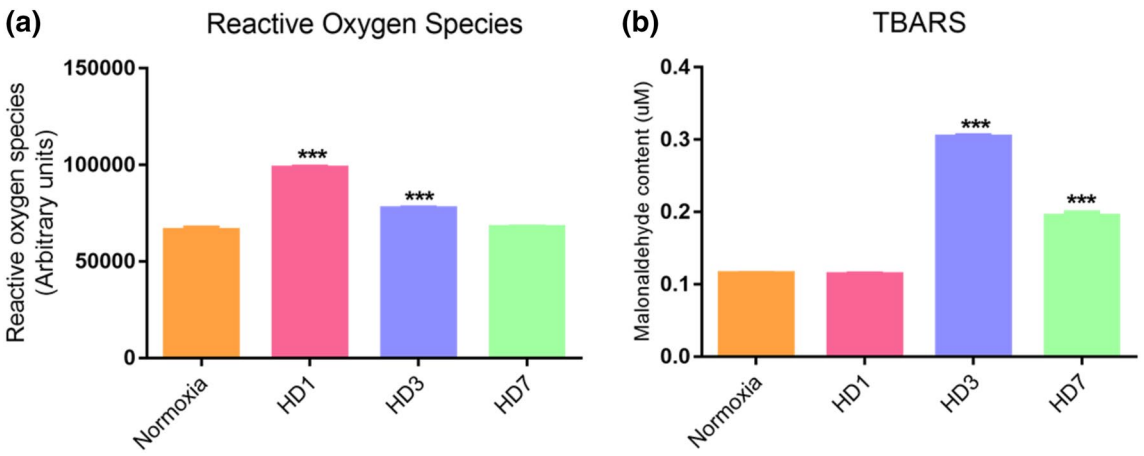

Fig. 1 Modulations in oxidative stress parameters in rat saliva. **a** Levels of reactive oxygen species were measured in rat saliva showing an increase in the levels of ROS at HD1 and HD3 groups in rat saliva as compared to N. **b** Lipid peroxides levels were measured and observed to be increased at HD3 followed by a decreased value in HD7 in rat saliva as compared to N. Results are expressed as Mean concentration ± SEM. Mean was calculated from three separate experiments (***represents $p < 0.001$ with respect to N)

controls to HD1 group which then increases till the HD7 group (Fig. 2b).

Nitrogen oxides are circulating vasoactive molecules that enable greater blood flow and oxygen delivery through vasodilation, thus, the levels of NOx were measured in rat saliva samples. The levels of NOx were found to be increased during hypoxia from normoxic controls to HD1 group and then decreased at HD7 group (Fig. 3).

Quantitative rat saliva proteome analysis using LC–MS/MS

Following the investigation of rat salivary proteome using iTRAQ labeled LC–MS/MS analysis, a huge list of proteins with quantification was obtained from LC–MS/MS to understand the casual events occurring in hypobaric hypoxia by simulated high altitude. Sixty-seven proteins were differentially expressed in the experimental groups as compared to normoxia (Table 1).

From 67 proteins, 36 (22 up-regulated and 14 down-regulated), 45 (24 up-regulated and 21 down-regulated), and 45 (19 up-regulated and 26 down-regulated) proteins were differentially expressed in HD1, HD3 and HD7 group respectively (Fig. 4a). 23 proteins were common between all the three groups while 5 proteins were common between HD1 and HD3 groups, 10 proteins were common between HD3 and HD7 and 5 proteins were common between HD1 and HD7 groups (Fig. 4b). Based on iTRAQ data, proteins such as BPI fold-containing family A member 2, cystatin and carbonic anhydrase 1 showed many folds differential expression in exposed groups as compared to normoxia. Upon pathway analysis through Ingenuity Pathway Analysis (IPA) software, we observed Glucocorticoid receptor signaling to be the most significant pathways based on p-value (Fig. 4c).

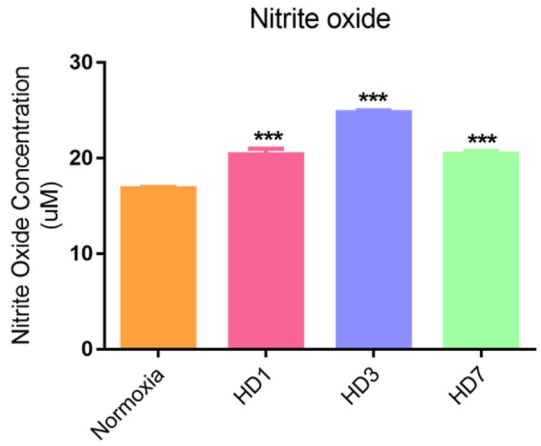

Fig. 3 Biochemical estimation of total nitrate + nitrite (NOx). Levels showing an increase in the levels of NO from N to HD3 group followed by a decreased value in HD7 groups as compared to N. Results are expressed as Mean concentration ± SEM. Mean was calculated from three separate experiments (***represents $p < 0.001$ with respect to N)

Further, the glucocorticoid receptor signaling pathway was investigated with overlaid differentially expressed proteins (Fig. 5a). The proteins involved were annexin 1, heat shock protein 90, and keratin. Their fold change values were represented in the form of a graph (Fig. 5b).

In glucocorticoid receptor signaling, annexin 1, also known as lipocortin 1 (possess anti-inflammatory properties) was observed to be decreased from normoxia to HD7 group. Another protein, hsp90, a chaperone protein was

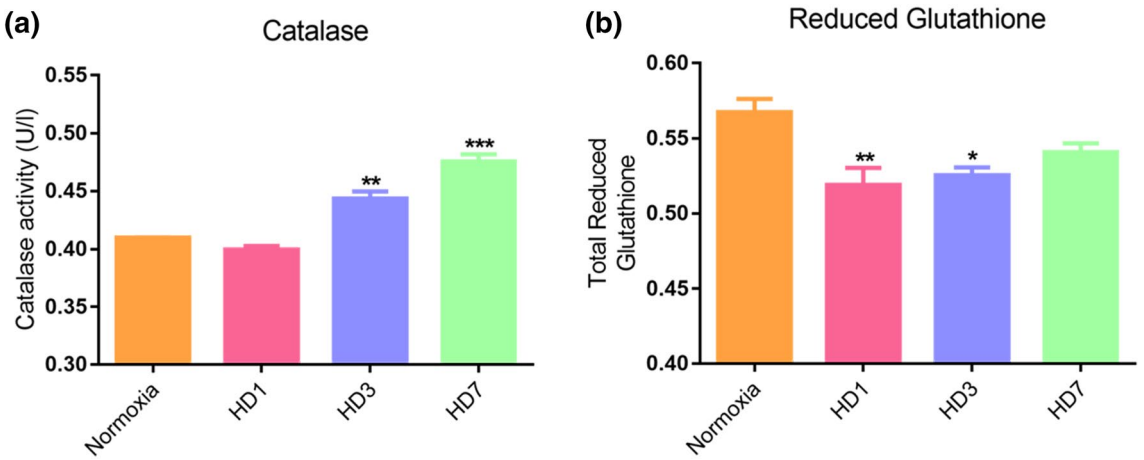

Fig. 2 Antioxidant status in response to hypobaric hypoxia. **a** Catalase activity was measured in rat saliva showing an increase in the levels of catalase activity at HD3 and HD7 groups as compared to N. **b** Levels of reduced glutathione were measured and observed to be decreased at HD1 followed by an increased value in HD3 till HD7 in rat saliva as compared to N. Results are expressed as Mean concentration ± SEM. Mean was calculated from three separate experiments (*represents $p < 0.05$, **represents $p < 0.01$ and ***represents $p < 0.001$ with respect to N)

Table 1 Overview of differentially expressed rat salivary proteins along with their Uniprot IDs and abundance ratios across HD1, HD3 and HD7 with respect to normoxic controls

Name of the Protein	Uniprot ID	Abundance ratio (HD1/N)	Abundance ratio (HD3/N)	Abundance ratio (HD7/N)
Alpha-amylase	Q99N59	2.802	5.398	10.083
Alpha-amylase	E9PSQ1	1.942	2.43	3.665
Carbonic anhydrase 6	F1LQ08	1.325	1.305	1.612
Gliadoralin-A	D3Z9M3	0.444	0.494	0.527
Deoxyribonuclease-1	P21704	1.904	1.912	2.604
Keratin 16	Q6IFU9	0.367	0.04	0.034
Cysteine-rich secretory protein 1	P12020	2.206	3.747	4.869
Keratin, type I cytoskeletal 14	Q6IFV1	0.447	0.086	0.045
Keratin, type I cytoskeletal 13	Q6IFV4	0.905	0.138	0.12
Keratin, type II cytoskeletal 4	Q6IG00	1.048	0.139	0.099
Keratin, type I cytoskeletal 10	A0A0G2K2V6	1.002	0.138	0.073
Common salivary protein 1	Q63015	0.745	0.685	0.852
Serum albumin	A0A0G2JSH5	0.871	0.964	0.557
Apomucin	Q62605	1.392	1.151	1.614
Lipoprotein(a)-like 2, pseudogene	D3ZVB6	1.744	4.313	1.356
Keratin, type II cytoskeletal 6A	Q4FZU2	0.765	0.137	0.131
Actin, cytoplasmic 1	A0A0G2K3K2	0.512	0.917	0.372
Prolactin induced protein, isoform CRA_d	G3V812	0.354	0.806	0.779
Globin c2	A0A0G2JSV6	0.844	2.82	0.981
Vomeromodulin	Q63751	3.047	6.361	1.933
Alpha-2-macroglobulin-like 1	D3ZS19	2.224	1.601	1.684
Keratin, type II cytoskeletal 2 epidermal	Q6IG02	1.02	0.529	0.441
Globin a4	A0A0G2JSW3	0.929	4.273	0.819
Keratin, type II cytoskeletal 1	A0A0G2JST3	1.269	0.168	0.054
Keratin 76	Q6IFZ5	2.557	0.195	0.131
von Ebner gland protein 1	P20289	0.834	1.05	1.247
Keratin, type II cytoskeletal 5	A0A091CUF8	0.386	0.054	0.03
Proline-rich 4	A0A0G2K5E1	0.526	0.39	0.732
Acidic mammalian chitinase	A0A091CUF8	1.484	2.878	2.659
Odorant-binding protein 1F	Q9QYU9	0.96	2.12	1.214
Odorant-binding protein	Q63613	4.113	5.055	3.108
Proline-rich 4	F1LSD9	0.547	0.602	0.866
Keratin, type II cytoskeletal 1 (Fragment)	G5ALS8	0.503	1.005	0.289
SMR2 protein	P18897	0.417	0.409	0.334
Ba1-667	Q7TP24	1.121	1.963	0.808
BPI fold-containing family A member 2	Q63471	2.571	8.128	7.764
BPI fold-containing family B member 1	A0A140TAH2	0.829	0.784	0.63
Kallikrein 6 (Predicted)	G3V8G8	3.578	6.575	2.409
Alpha-2u globulin	Q63213	2.404	2.635	2.822
Cystatin	D4AAU9	2.317	2.925	4.249
Adenosine deaminase	Q920P6	1.276	0.37	0.162
LOC500183 protein	Q4KM66	0.817	0.672	0.621
Androgen binding protein, alpha	G3V9B4	2.183	2.099	3.088
Kallikrein 1-related peptidase B3	G3V8H1	1.433	1.863	1.167
Complement factor B	Q7TP05	1.119	1.169	0.901
Nucleobindin 2, isoform CRA_b	G3V8R1	2.136	1.795	1.588
Kallikrein a4	A0A1R3UCJ4	1.28	1.111	1.087
Annexin A1	P04083	0.682	0.43	0.138

Table 1 (continued)

Name of the Protein	Uniprot ID	Abundance ratio (HD1/N)	Abundance ratio (HD3/N)	Abundance ratio (HD7/N)
BWK3	Q5VLR6	1.285	0.826	0.607
BPI fold-containing family A, member 2F	Q63550	0.533	0.257	0.4
Alpha-1-antiproteinase	A0A0G2JZ73	0.959	0.608	0.446
Inter-alpha-trypsin inhibitor heavy chain H3	D3ZBS2	0.646	0.861	0.431
Peptidyl-glycine alpha-amidatingmonooxygenase	P14925	1.591	0.808	1.057
Serpin family B member 12	D3ZPF9	1.527	0.393	0.515
116 kDa U5 small nuclear ribonucleoprotein component	A0A091CZK5	0.976	0.42	0.366
Carbonic anhydrase 1	B0BNN3	0.644	3.421	0.759
Alpha-enolase	A0A091D5P2	1.359	1.081	0.755
Salivary lipocalin	G5BWR1	1.127	1.21	1.499
Lipocalin 3	D3ZK46	2.316	1.571	0.942
Ig gamma-2B chain C region	P20761	3.223	4.661	1.655
ABP beta (Fragment)	Q5I1B4	1.661	1.327	1.72

found to be normalized in HD1 followed by a decreased expression in HD3 and HD7 groups. Fold change values of different types of keratin cytoskeletal proteins were taken into account through IPA and it was observed that in HD1 group, KRT16 and KRT13 showed decreased expression; KRT14 and KRT4 were normalized; and KRT1 and KRT76 showed increased expression as compared to normoxia. In HD3 and HD7 groups, all types of keratin showed decreased expression as compared to normoxia.

Another important pathway observed was MSP-RON signaling. This pathway was significantly modulated in the HD1 group and investigated further for exploring significant proteins having differential expression in the exposure groups as compared to normoxia (Fig. 6). Two of the proteins were found to be involved in this pathway such as actin and kallikrein 1. In terms of expression, actin showed decreased expression in HD1 and HD7 while it was normalized at the HD3 group of exposure. And, kallikrein 1 showed increased expression from normoxia to HD3 group followed by a decrease in HD7 group.

Discussion

Hypobaric hypoxia is known to be associated with oxidative stress and leads to various clinical manifestations such as acute mountain sickness (AMS), high altitude pulmonary edema (HAPE) and high altitude cerebral edema (HACE) and non-clinical conditions such as acclimatization and adaptation. Proteomic markers have been studied during these pathophysiological and non-pathological conditions indicating a strong relationship between apparent changes and underlying molecular mechanisms (Ahmad et al. 2011, 2013, 2014; Jefferson et al. 2004; Magalhaes et al. 2005; Padhy et al. 2013; Sharma et al. 2013; Vij et al. 2005; Gaur et al. 2018; Jain et al. 2018, 2019). Previously suggested, oxidative stress involves the activation of inflammatory pathways that were found to be associated with various conditions such as cancer, cardiovascular, neurological, and pulmonary pathologies (Reuter et al. 2010). The differential expression of proteins holds promise for the assessment of underlying mechanisms based on putative protein markers, but, most of the proteins detected earlier were either systemic or specific to particular tissues and the detection methods were invasive. Also studies focussed on the effect of hypobaric hypoxia before seven days are limited. Therefore, the focus shifted to a non-invasive approach wherein saliva samples could be utilized using an animal model.

In this study, an important aspect, redox homeostasis, a key process in hypoxia has been explored in saliva through estimation of oxidative stress parameters and anti-oxidants levels using biochemical assays in the experimental groups from N to HD7. Reactive oxygen species were found to be highest in the HD1 group. Also, lipid peroxides were estimated and observed to be increased at HD3 followed by normalization at HD7. Another marker, nitric oxide, responsible for vasodilation during hypoxia, was increased from HD1 to HD3 followed by normalization at HD7. Antioxidant enzymes such as catalase and reduced glutathione showed opposite patterns to oxidative stress indicators. Earlier reports suggested oxidative stress occurs due to the imbalance between prooxidants and antioxidants which have been proved by our study also (Coimbra-Costa et al. 2017; Debevec et al. 2017).

Also, we have found the maximum number of differentially expressed proteins in HD3 and HD7 groups. Based on iTRAQ data, proteins such as BPI fold-containing family A member 2, cystatin and carbonic anhydrase 1 showed

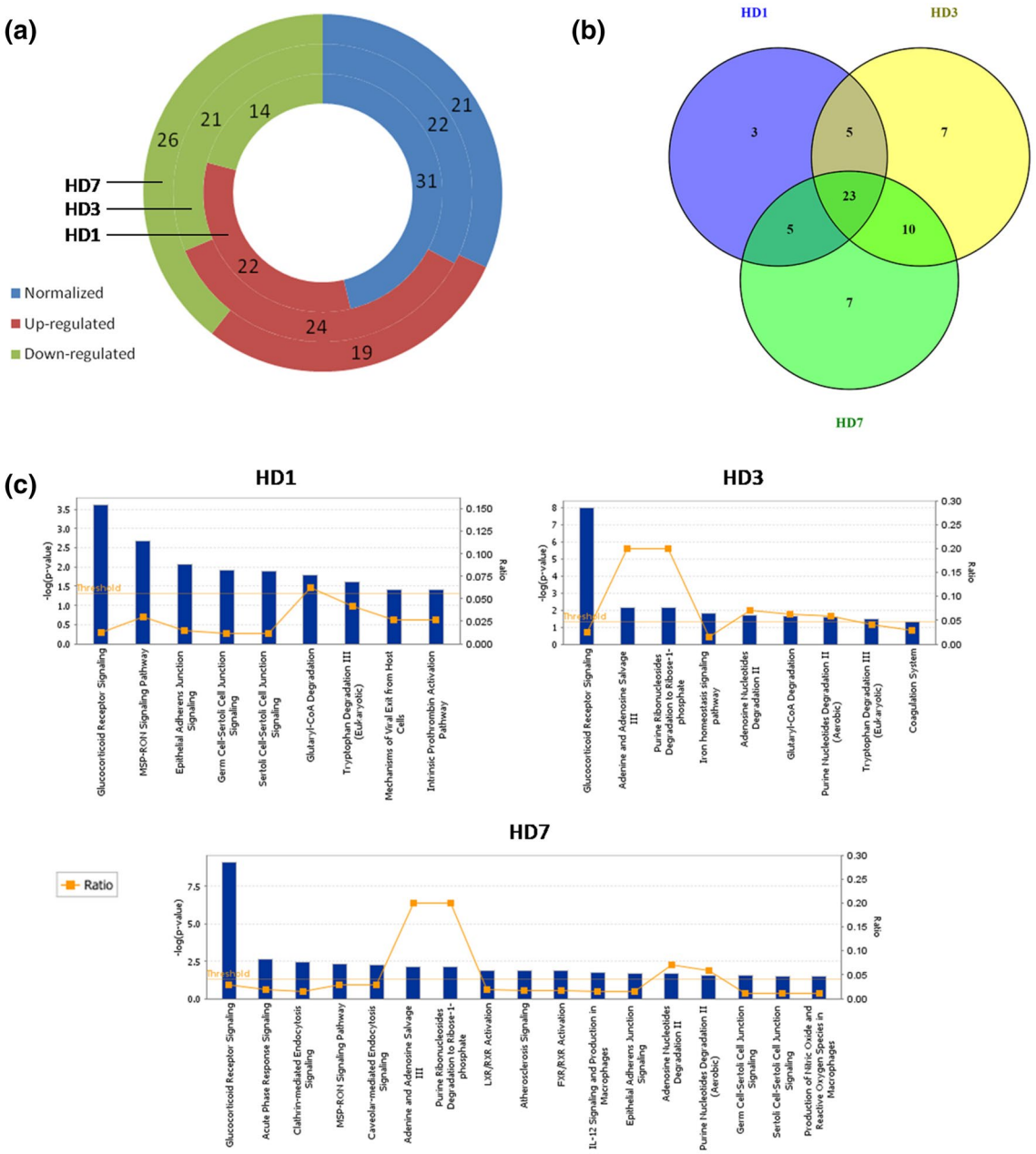

Fig. 4 Overview of rat salivary proteome along with affected pathways. **a** The total number of up-regulated (red) and down-regulated (green) proteins along with normalized proteins (blue) identified in HD1, HD3, and HD7 groups during LCMS/MS (iTRAQ labeled) analysis of rat saliva. Abundance ratio value greater than 1.5 fold was considered up-regulation while value lesser than 0.67 was considered as down-regulation. **b** Venn diagram of overlapping up- and down-regulated proteins among the proteins in HD1(blue), HD3 (yellow) and HD7 (green) groups using Oliveros, J.C. (2007–2015) Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html. **c** Ingenuity pathway analysis (IPA) of rat salivary proteins revealing Glucocorticoid receptor signaling to be the most significant pathways in HD1, HD3 and HD7 groups based on p value < 0.001

many folds differential expression in exposed groups as compared to normoxia. BPI fold-containing family A member 2 is a protein highly up-regulated in the exposure groups as compared to normoxic controls. Earlier researchers suggested this protein as an early biomarker of acute kidney injury (Honore et al. 2018; Kota et al. 2017). Increased expression of other proteins closely linked to cystatin and carbonic anhydrase 1 have already been proved by our previous studies in response to hypobaric hypoxia in the human model (Jain et al. 2018, 2020).

Upon IPA, the most significantly modulated pathway was glucocorticoid receptor signalling. In glucocorticoid

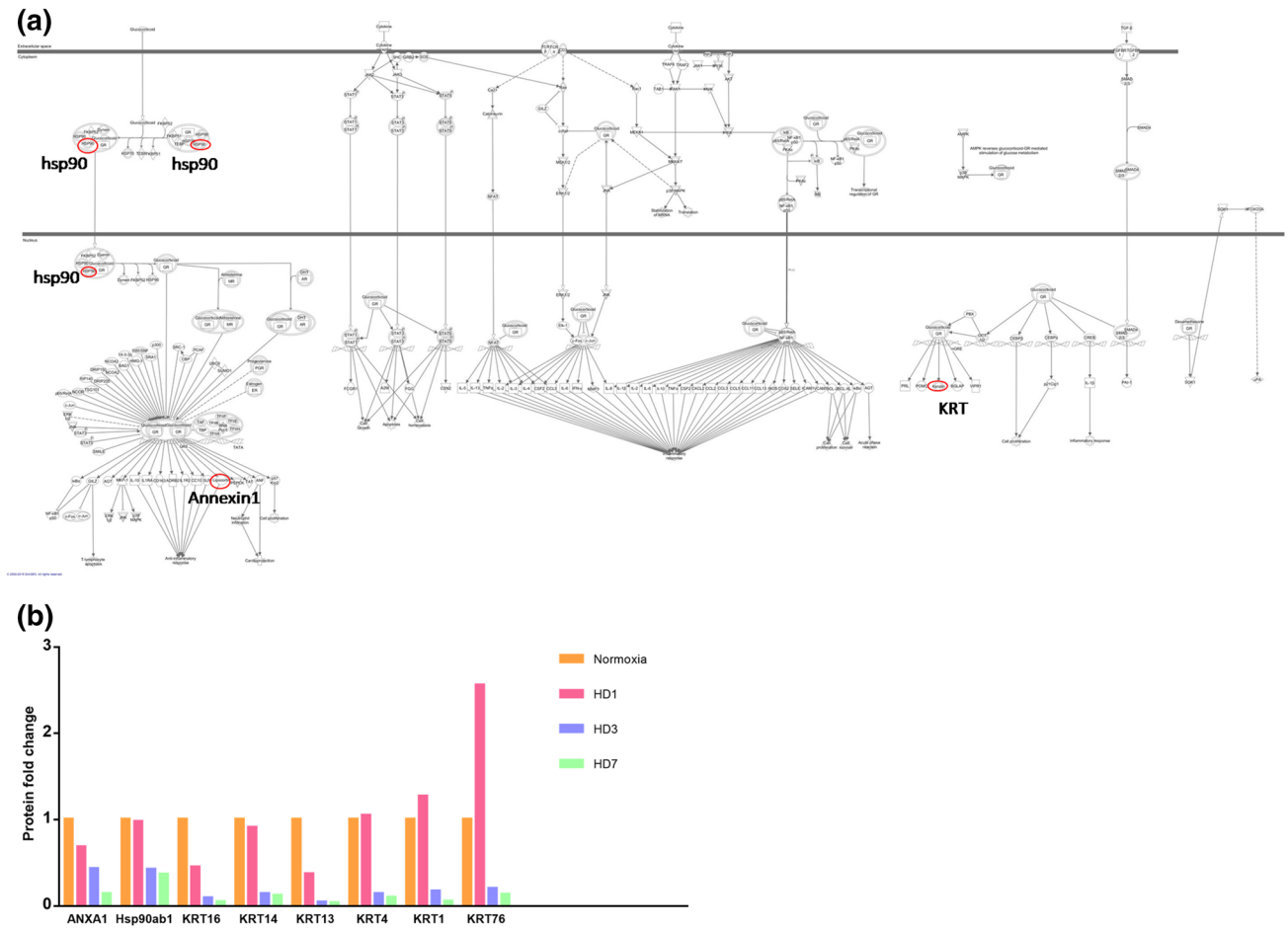

Fig. 5 Glucocorticoid receptor signaling pathway. **a** Overview of the pathway with overlaid expressed saliva proteins, **b** fold change values of key proteins involved in the pathway (p value < 0.05)

receptor signalling pathway, major differentially modulated proteins were annexin 1, heat shock protein 90, and keratin. Previously, researchers suggested that inhibition of glucocorticoid receptors may provide protection to HH induced memory impairment (Baitharu et al. 2013). In terms of differentially expressed proteins, annexin 1, an immunomodulatory and anti-inflammatory protein plays a role as homeostatic protein that regulates essential transcription factors and miRNAs. It has been observed to be a clinical marker for gingivitis in pregnant women, coronary heart disease (Hassan et al. 2018; Sarndahl et al. 2010). Annexin A1 has also been found to be a potential marker and therapeutic agent for asthma using plasma as a biological fluid (Lee et al. 2018). Another protein, hsp90, a cytoprotective chaperone was recently recommended as a potential biochemical marker for screening of individuals susceptible or tolerant to acute hypobaric hypoxia (Jain et al. 2013). This molecular chaperone facilitates protein maturation, stabilization of aggregation-prone proteins, quality control of misfolded proteins, and assists in keeping proteins in activation-competent conformations. Many of the proteins chaperoned by Hsp90 (Hsp90 clients) are essential for the progression of various diseases, including cancer, Alzheimer's disease, and other neurodegenerative diseases, as well as viral and bacterial infections (Zuehlke et al. 2018). Also, it helps in mediating differential hypoxic tolerance by enhancing the ability to sustain survival under hypobaric hypoxia (Jain et al. 2014). Previous studies also demonstrated that plasma Hsp90α protein levels are useful as a diagnostic biomarker in lung cancer and acute lymphoblastic leukemia (ALL) (Shi et al. 2014; Milani et al. 2015). A study by St. Pierre Schneider B et. al. suggested, the third set of proteins; keratins, expressed in the differentiated epithelial layers of skin, oral cavity and squamous stomach involved in epithelial homeostasis have shown to be down-regulated in hypobaric hypoxia caused by long-distance air travel (Schneider et al. 2013; Sequeira et al. 2018). Also, Srikant Ambatipudi et. al. suggested down-regulation of krt76 is linked to oral carcinogenesis (Ambatipudi et al. 2013).

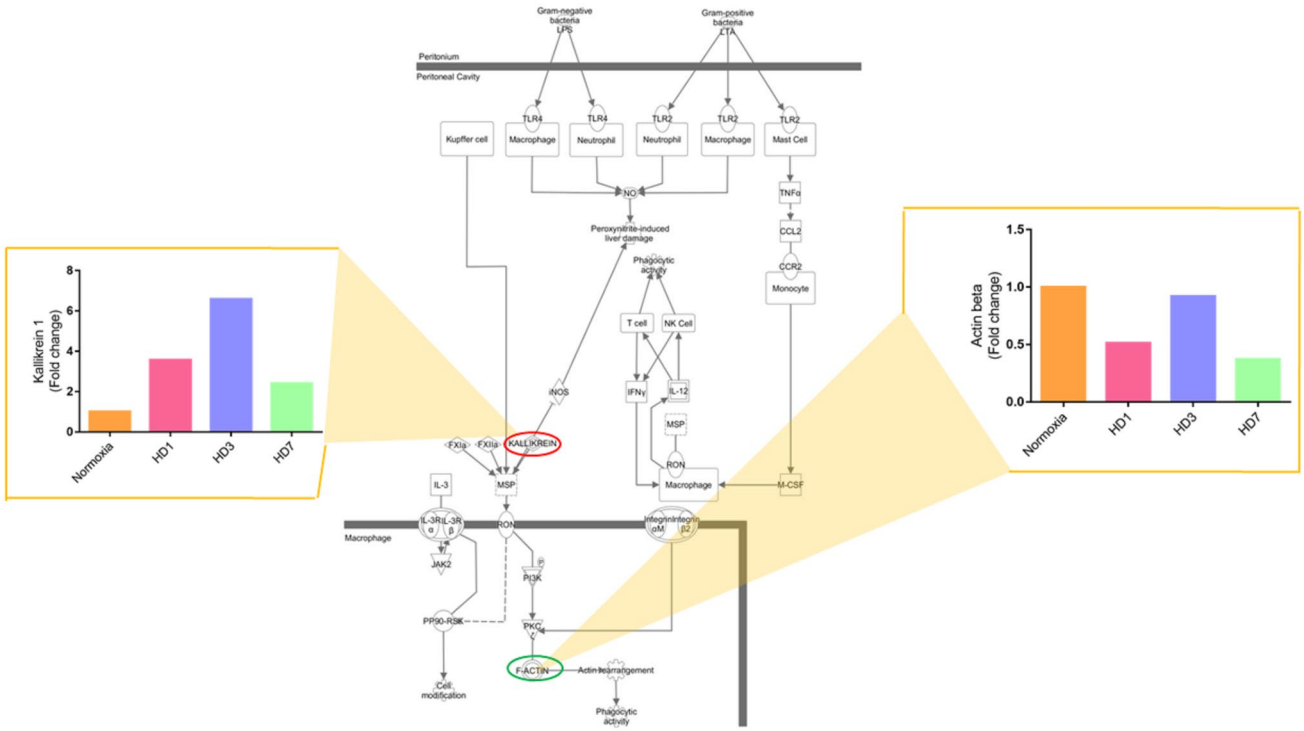

Fig. 6 MSP-RON signaling pathway. Overview of the pathway with overlaid expressed saliva proteins along with their fold change values (p value < 0.05)

Another significant pathway observed was MSP-RON signalling. Earlier researchers have recommended the MSP-RON pathway as a therapeutic target for a variety of cancers because of its role in the pathogenesis of cancer. This pathway has been extensively studied in various experimental model systems (Yao et al. 2013). In MSP-RON signalling, two proteins such as actin and kallikrein 1 were differentially expressed in exposure groups as compared to normoxic controls. Previously, actin, a cytoskeletal protein has been observed to be down-regulated during hypobaric hypoxia and HAPE (Hernandez et al. 2013; Mairbaurl et al. 1985; Peinado et al. 2014). A recent study by Li K et. al. suggested over-expression of kallikrein 1, a serine protease may be closely linked to hypertension, inflammation, obesity and other gastric injuries associated with high altitude-induced polycythemia (Li et al. 2017).

Our observations also coincide with the earlier reports that suggested these pathways and involved proteins may have a role in regulating/modulating the hypoxic response and may help in the proteome based assessment of hypobaric hypoxia.

Conclusion

In conclusion, this study provided proof of concept for understanding hypobaric hypoxia-mediated events through rat salivary proteome. Glucocorticoid receptor signaling and MSP-RON signalling are the most significant pathways involving modulated proteins such as annexin 1, hsp90, keratin, actin-beta, and kallikrein 1. These proteins were found to aid in acclimatization and survival during hypobaric hypoxia.

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Author contributions Conceived and designed the experiments by YA and KB. Experiments were performed by SJ, SP and AG. Representative figures were designed by SJ. Manuscript was written by SJ. Manuscript was critically evaluated by YA and KB.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest in this work.

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