Changes in the Urinary Proteome in a Rat Model of Acute Hypoxia

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Abstract

[Objective] This study aims to investigate the effects of hypoxia on the rat urinary proteome and identify changes in urinary proteins associated with hypoxic stress.

[Methods] This study employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen for differential urinary proteins in rat models of 12-hour and 24-hour hypoxia and performed analysis of biological pathways to observe changes in urinary proteins during acute hypoxic stress.

[Results] We found that the urinary proteome could clearly differentiate samples from the normoxia and hypoxia groups, and differential proteins were also enriched in biological pathways related to hypoxic stress, such as antioxidant stress response, glycolysis, complement and coagulation cascades, etc.

[Conclusion] The urinary proteome can reflect significant changes following acute hypoxic stimulation and can assist in detecting hypoxic status.

Full Text

Changes in the Urine Proteome of an Acute Hypoxic Rat Model

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Abstract

[**Objective**] To investigate the effects of hypoxia on the urine proteome in rats and identify urine protein changes associated with hypoxic stress.
[**Methods**] This study employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen for differential urine proteins in rat models of 12-hour and 24-hour hypoxia, followed by biological pathway analysis to observe urine protein changes during acute hypoxic stress.
[**Results**] We found that the urine proteome could clearly distinguish normoxic from hypoxic samples, with differential proteins enriched in biological pathways related to hypoxic stress, such as antioxidant stress, glycolysis, and complement and coagulation cascades.
[**Conclusions**] The urine proteome can reflect significant changes following acute hypoxic stimulation, which may aid in the detection of hypoxic states.

Keywords: proteomics; urine; hypoxia

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High-altitude regions are characterized by low temperatures, large temperature variations, and strong ultraviolet radiation [1], with low pressure and hypoxia being the most prominent features [2]. Medically, "high altitude" typically refers to areas above 3000 meters. As altitude increases, atmospheric oxygen partial pressure gradually decreases, causing a sharp decline in human blood oxygen saturation and the frequent appearance of hypoxic symptoms [3]. Globally, nearly 140 million people reside in plateau regions above 2500 meters, with approximately 40 million people traveling to high-altitude areas annually for work or leisure [4]. In China alone, 60 million people live in vast plateau regions [5]. This unique geographical environment endows high-altitude populations with distinct physiological characteristics. Compared to low-altitude populations, they exhibit increased brain oxygen consumption, elevated pulmonary ventilation and alveolar oxygen partial pressure, and excessive erythrocytosis and hematocrit elevation caused by hypoxia, which leads to increased blood viscosity and reduced flow velocity, substantially raising the risk of thrombosis. Moreover, hypoxia represents the greatest challenge of high-altitude environments. Failure to adapt to high altitude can result in various altitude-related illnesses,

ranging from mild symptoms such as nausea, dizziness, insomnia, palpitations, and shortness of breath to life-threatening conditions including high-altitude pulmonary edema (HAPE), high-altitude polycythemia (HAPC), and high-altitude cerebral edema (HACE) [6]. We aim to dynamically monitor the progression of hypoxic injury in high-altitude populations and accurately assess the degree of altitude hypoxia in the body to enable timely and precise treatment for patients. Furthermore, a better understanding of cellular and organ functional changes induced by hypoxia is essential and necessary for developing new physiological and pathological candidates to reveal the underlying pathogenic mechanisms of high-altitude hypoxia and improve treatments for hypoxia-related diseases.

Disease progression involves different biological processes that exhibit distinct pathophysiological states at various time points, suggesting that different biomarkers should be present at different stages. Urine represents an ideal source of disease biomarkers because, lacking homeostatic regulatory mechanisms, it can sensitively and timely reflect pathological changes [7]. Our laboratory has previously discovered in multiple disease animal models using proteomics technology that urine proteins change before pathological alterations appear [8], demonstrating that the urine proteome can capture early changes produced by the body. Additionally, urine collection is non-invasive, simple, and can be performed continuously, enabling dynamic disease monitoring.

In this study, we simulated high-altitude hypoxic conditions at 5000 meters using a hypoxic chamber and performed label-free quantitative proteomics analysis of urine collected at 0, 12, and 24 hours after hypoxia exposure. We aimed to investigate the impact of hypoxic conditions at 5000 meters altitude on the rat urine proteome and identify urine protein changes associated with hypoxic stress, providing a preliminary exploration for subsequent identification of early, specific biomarkers for hypoxia in urine.

1.1 Experimental Animals and Model Establishment

Metabolic Cage Modification
Rats were placed in modified mouse-sized metabolic cages for urine collection. The cages were fitted with a sieve filter (15 cm diameter, 1 cm mesh spacing) to remove food residues and feces.

Hypoxia Model Establishment
Five SPF-grade male Sprague-Dawley rats (170–190 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed in a standard environment (temperature (22$±$1)°C, humidity 65%–70%), and animal experiments were reviewed and approved by the Life Sciences Ethics Committee of Beijing Normal University.

Modeling Method: Rats were placed in a hypoxic chamber with oxygen concentration set at 11.6% to simulate hypoxic conditions at 5000 meters altitude. This experiment used self-control, where urine samples collected under normoxic conditions served as the control group (time point T0). Urine samples collected

after 12 hours and 24 hours of hypoxia were designated as experimental groups (time points T1 and T2, respectively).

1.2 Urine Collection

Each rat was individually placed in a modified metabolic cage. Sampling time points were set at T0 (0 hours), T1 (12 hours), and T2 (24 hours) under hypoxic conditions. During urine collection, water was provided but food was withheld to avoid contamination. A total of 15 samples were collected. Urine was centrifuged at 3000×g for 30 minutes and stored at −80°C.

1.3 Urine Protein Extraction and Proteolysis

Urine samples (4 mL) were thawed and centrifuged at 12,000×g for 30 minutes at 4°C to remove cell debris. The supernatant was precipitated with 3 volumes of ethanol overnight, then centrifuged at 12,000×g for 30 minutes. Protein pellets were resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, and 50 mmol/L Tris). Protein concentration was measured using the Bradford method. Urine protein digestion was performed using the filter-aided sample preparation (FASP) method [9]. Urine proteins were loaded onto 10 kDa ultrafiltration tubes (PALL), washed twice with UA (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) and 25 mmol/L NH_4HCO_3 solution, denatured with 20 mmol/L dithiothreitol (DTT, Sigma) at 37°C for 1 hour, alkylated with 50 mmol/L iodoacetamide (IAA, Sigma) in the dark for 30 minutes, washed twice with UA and NH_4HCO_3 solution, and digested with trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) at a 1:50 ratio at 37°C overnight. After overnight incubation, the digested filtrate was collected by centrifugation as the peptide mixture.

Peptides were desalted using HLB columns (Waters, Milford, MA), vacuum-dried, and stored at −80°C.

1.4 LC-MS/MS Tandem Mass Spectrometry Analysis

Peptides were resuspended in 0.1% formic acid, and concentration was measured using a BCA assay kit and diluted to 0.5 g/ L. One microgram of peptide sample was separated using a Thermo EASY-nLC1200 liquid chromatography system with a 90-minute elution gradient (mobile phase A: 0.1% formic acid; mobile phase B: 80% acetonitrile). Eluted peptides were detected using an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA). Data-dependent acquisition (DDA) mass spectrometry data were collected for all samples, with three technical replicates per sample.

1.5 Data Analysis

Raw data (RAW files) obtained from LC-MS/MS were analyzed using Proteome Discoverer (version 2.1, Thermo Scientific) and MaxQuant (version 1.6.17.0).

Base peak chromatograms were examined using Xcalibur Qual Browser (version 3.0.63, Thermo Fisher Scientific). RAW files were processed in MaxQuant using mostly default parameters. All RAW files were processed in the same MaxQuant window, with database searches performed using the Andromeda search engine (configured in MaxQuant) against the UniProt Rattus norvegicus (Rat) sequence database (March 17, 2020; 8,137 sequences). Precursor mass tolerance was set to 4.5 ppm for the main search, and fragment mass tolerance was set to 20 ppm. Digestion enzyme was set to Trypsin/P with a maximum of two missed cleavages. Minimum peptide length was set to seven residues. Protein N-terminal acetylation and methionine oxidation were set as variable modifications, while cysteine carbamidomethylation was set as a fixed modification. Following reduction and alkylation, disulfide bonds are opened and cysteine residues carry a carbamidomethyl group (+57 Da), which is typically selected as a fixed modification (C) during database searching. No Andromeda score threshold was set for unmodified peptides; each identified modification required a minimum Andromeda score of 40. Peptide and protein false discovery rates were set to 1% based on a target-decoy reverse database. Elution times were aligned between samples using the "match between runs" option (match time window: 0.7 min, alignment time window: 20 min). Label-free quantification (LFQ) was enabled using the MaxLFQ algorithm from MaxQuant. Protein LFQ intensities were calculated as the median of pairwise intensity ratios from peptides identified in two or more samples, adjusted according to cumulative intensity across samples. Quantification was performed using razor and unique peptides, including those with acetylation (protein N-terminus) and oxidation (Met) modifications. Protein intensity normalization required a minimum peptide ratio of 1, and "Fast LFQ" was enabled.

Data were subsequently preprocessed using Perseus (version 1.6.14.0). Contaminants and proteins with fewer than one identified peptide were removed. LFQ intensities were $\log_2$-transformed for normalization. Missing values were considered to represent low-abundance proteins below the detection limit of mass spectrometry rather than random missingness. To simulate low-abundance LFQ values, missing values were replaced with random values from a normal distribution below the median and then set to 0 [10,11].

1.6 Statistical Analysis

Each sample was analyzed with three technical replicates, and the resulting data were used for statistical analysis. Urine proteins identified at hypoxic time points T1 and T2 were compared with those identified at normoxic time point T0 to screen for differential proteins. Screening criteria were as follows: fold change $ $2 or $ $0.5, and two-tailed unpaired t-test P-value <0.01.

1.7 Random Grouping Analysis

To address the possibility that the high dimensionality of proteomic features relative to sample size might produce random differences between groups, we

developed a random grouping statistical strategy to confirm whether differential proteins were disease-related. Hypoxic T1 group samples (n=5) and normoxic T0 group samples (n=5) were randomly divided into two groups. Across 126 total random combinations, the average number of differential proteins identified using the same screening criteria was 5 (see Supplementary Table 1). These results indicate that only 5 differential proteins could be generated randomly, further demonstrating that 96.5% of the identified differential proteins are reliable. The same procedure was applied to T2 and T0 group samples, yielding an average of 6 randomly generated differential proteins (see Supplementary Table 2).

1.8 Bioinformatics Analysis

Unsupervised hierarchical clustering analysis (HCA) was performed using the Wukong platform (https://www.omicsolution.org/wkomic/main/) [12]. Functional enrichment analysis of differential proteins identified across the three models was conducted for biological process, cellular component, and molecular function using DAVID 6.8 (https://david.ncifcrf.gov/) [13]. The functions of differential proteins were investigated based on reports from publicly available databases (https://pubmed.ncbi.nlm.nih.gov).

2.1 Urine Proteome Changes in the Hypoxia Model

In the hypoxia model, urine proteins from 5 rats at T1 and T2 time points were identified using label-free LC-MS/MS. A total of 1,162 proteins ($ $2 unique peptides, protein-level FDR<1%) were identified. Unsupervised hierarchical clustering analysis clearly distinguished normoxic from hypoxic samples. Figure 1 shows the detailed unsupervised clustering results for the samples.

Using a self-control approach, urine proteins at T1 and T2 were compared with those at T0. Screening criteria were: fold change FC$ $2 or $ $0.5, P<0.01, average spectral count in the high-abundance group $ $3, and spectral counts for each sample in the high-abundance group higher than those in the low-abundance group. Compared with T0, 144 differential proteins were identified at T1, including 88 upregulated and 56 downregulated proteins. At T2, 129 differential proteins were identified, including 73 upregulated and 56 downregulated proteins.

Figure 1. Unsupervised hierarchical clustering analysis of the overall urine proteome from hypoxic and normoxic groups. Green labels (T0) represent normoxic samples, pink labels (T1) represent 12-hour hypoxic samples, and light blue labels (T2) represent 24-hour hypoxic samples. The horizontal axis shows unsupervised clustering and sample details, while the vertical axis shows specific protein names.

2.2 Random Grouping Results for Urine Samples

Given that the number of proteomic features identified exceeded the number of samples, differences between groups might arise randomly. We developed a random grouping statistical strategy to confirm whether these differential proteins were disease-related. Hypoxic T1 group samples (n=5) and normoxic T0 group samples (n=5) were randomly divided into two groups. Across 126 total random combinations, the average number of differential proteins identified using the same screening criteria was 5 (see Supplementary Table 1). These results indicate that only 5 differential proteins could be generated randomly, further confirming that 96.5% of the differential proteins are reliable. The same procedure was applied to T2 and T0 group samples, yielding an average of 6 randomly generated differential proteins (see Supplementary Table 2).

2.3 Functional Annotation of Differential Proteins

DAVID database was used to perform functional enrichment analysis of the 144 differential proteins identified at T1 across three aspects: biological process, cellular component, and molecular function (Figure 2). In biological processes (Figure 2A), these differential proteins were enriched in glutathione metabolism, negative regulation of endopeptidase activity, angiogenesis, cellular oxidative detoxification, and glycolysis. In cellular components (Figure 2B), most differential proteins originated from extracellular exosomes and extracellular space. In molecular functions (Figure 2C), these proteins were enriched in cadherin binding involved in cell-cell adhesion, calcium ion binding, protein binding, and glutathione transferase activity. To identify major metabolic pathways involved, KEGG pathway enrichment analysis was performed, revealing 9 significantly enriched pathways including glutathione metabolism, nitrogen metabolism, and carbon metabolism (Figure 2D).

Similarly, DAVID database was used to analyze the 129 differential proteins identified at T2 (Figure 3). In biological processes (Figure 3A), these proteins were enriched in negative regulation of endopeptidase activity, cell adhesion, platelet aggregation, and blood coagulation. In cellular components (Figure 3B), most differential proteins originated from extracellular exosomes and extracellular space. In molecular functions (Figure 3C), these proteins were enriched in cadherin binding involved in cell-cell adhesion, collagen binding, and carbonate dehydratase activity. KEGG pathway enrichment analysis revealed 3 significantly enriched pathways: nitrogen metabolism, platelet activation, and regulation of actin cytoskeleton (Figure 3D).

Figure 2. Functional annotation of differential proteins at T1. A. Biological process; B. Cellular component; C. Molecular function; D. Biological pathways.

Figure 3. Functional annotation of differential proteins at T2. A. Biological process; B. Cellular component; C. Molecular function; D. Biological pathways.

Following bioinformatics analysis using DAVID and KEGG databases, several

biological processes and metabolic pathways were found to be reported in association with hypoxia: (i) **Antioxidant stress.** Studies have reported that oxidants induced by hypobaric hypoxia regulate the activity of two major antioxidant protein families: peroxiredoxins (PDX) and thioredoxins [14]. Members of the thioredoxin superfamily contain a thioredoxin fold and a conserved active site sequence (CxxC), playing roles in redox regulation, oxidative stress defense, refolding of disulfide-containing proteins, and regulation of transcription factors [15]. (ii) **Glycolysis.** As O_2 levels decrease at high altitudes, ATP production shifts from oxygen-dependent oxidative phosphorylation in mitochondria to oxygen-independent glycolysis in the cytoplasm [16]. (iii) **Complement and coagulation cascades.** Research has found that plasma levels of coagulation-related proteins are higher in high-altitude populations than in plain-dwelling populations, indicating altered coagulation system regulation [17]. (iv) **Angiogenesis.** Hypoxia causes increased accumulation of HIF-1α (hypoxia-inducible factor 1), thereby inducing elevated expression of vascular endothelial growth factor and enhanced angiogenesis [18]. (v) **Renin-angiotensin system.** Studies have shown that activation of the renin-angiotensin system is associated with chronic kidney hypoxia [19].

Comparing the enriched biological processes between the two time points revealed that after 12 hours of hypoxia, changes were primarily manifested in altered substance metabolism, such as glutathione metabolism and glycolysis. After 24 hours of hypoxia, the body' s response shifted from metabolic changes toward organic changes such as angiogenesis and coagulation.

This study established an acute hypoxic rat model and used label-free LC-MS/MS to investigate the impact of hypoxic conditions at 5000 meters altitude on the rat urine proteome, aiming to understand the body' s changes during hypoxia adaptation and lay the foundation for identifying hypoxia biomarkers and therapeutic targets for hypoxemia-related diseases. The results showed that 144 differential proteins were identified after 12 hours of hypoxia and 129 after 24 hours, with 104 proteins common to both time points. This indicates that exposure to hypoxic environments induces acute mountain sickness and physiological changes. Subsequently, with prolonged exposure, the body mobilizes a series of compensatory hypoxia regulatory responses—including increased pulmonary ventilation [20], increased red blood cells and hemoglobin [21], and altered energy metabolism [22]—to improve oxygen supply and utilization, restore homeostasis, and adapt to the high-altitude hypoxic environment, a process known as altitude acclimatization.

Furthermore, most of these differential proteins showed "presence-to-absence" or "absence-to-presence" changes. In our previous studies on hyperlipidemia, tumor injection [23], pulmonary fibrosis [24], and Alzheimer' s disease [25], no pathophysiological changes have produced such profound effects on urine proteins. This demonstrates that the impact of hypoxia on the body is remarkably significant in the urine proteome, highlighting the sensitivity of urine as a biomarker source.

Our findings demonstrate that the urine proteome can reflect significant changes following acute hypoxic stimulation. These results may provide a method for assessing the degree of altitude hypoxia in the body, helping to detect or assist in detecting hypoxic states, thereby facilitating early implementation of appropriate therapeutic measures. This approach is non-invasive, readily accepted by patients, and particularly suitable for dynamic monitoring of hypoxic injury progression in high-altitude populations.

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Author Contributions

Youhe Gao, Ming Fan, Lingling Zhu, Yijin Bao, and Xiang Cheng: Provided samples, conceived the research idea, and designed the study protocol.
Yijin Bao and Xiang Cheng: Performed the experiments.
Yijin Bao: Analyzed the data.
Yijin Bao: Drafted the manuscript.
Youhe Gao: Revised the final manuscript.

Note: Figure translations are in progress. See original paper for figures.