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Research Article

Transcriptional Profiling in the Intestinal of Rats under Hypobaric Hypoxia

Jiayu Xu[1*#]; Chengliang Yin[2,3#]; Ming Lv[2]; Da Li[1]

[1]Graduate School of People's Liberation Army General Hospital, Beijing, China

[2]National Engineering Research Center for Medical Big Data Application Technology, Chinese PLA General Hospital, Beijing 1000853, China

[3]Department of Medical Engineering, Medical Supplies Center of PLA General Hospital, Beijing 1000853, China

***Corresponding author: Jiayu Xu**

Graduate School of People's Liberation Army General Hospital, Beijing, China.

Email: jiayxu301@163.com

[#]These authors have been equally contributed to this article.

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Abstract

High-altitude ascent induces various physiological changes, with Gastrointestinal (GI) disorders being a prevalent occurrence. The gut plays a pivotal role in maintaining overall body homeostasis during normal physiological adaptation. The molecular and pathophysiological mechanisms underlying gastrointestinal injury resulting from exposure to a hypoxic environment remain largely unknown. To comprehensively explore the potential physiological changes associated with intestinal disorders after exposure to a hypobaric hypoxic environment, we employed genome-wide transcriptional profiling to examine gene expression alterations in the small intestine of rats. Our study involved RNA-Sequencing (RNA-Seq) of the intestinal tissues of rats exposed to simulated hypobaric hypoxia for 2 weeks (W2Z) and 4 weeks (W4Z), allowing us to investigate the transcriptional profile during both acute and chronic hypobaric hypoxic conditions in this animal model. We identified differentially expressed genes among the three groups. A principal component analysis revealed substantial distinctions between the acute and chronic hypobaric hypoxia groups when compared to the control group. Furthermore, pathway analysis indicated that the differentially expressed genes were associated with the interaction of neuroactive ligands and receptors. This suggests that the adaptation of rats to hypobaric hypoxia involves, at least partially, the regulation of the neuroactive ligand-receptor interaction pathway.

Keywords: Hypobaric hypoxia; Small intestine; Transcriptional analysis; Rat model

Introduction

When humans ascend to high altitudes, the lower partial pressure of inspired oxygen results in reduced blood oxygen content, a condition known as systemic hypoxemia. Consequently, tissues, including the small intestine, experience diminished oxygen availability [17]. Exposure to high altitudes, particularly at elevations above 2500 meters, can lead to symptoms such as headaches and other manifestations [10,19].

One common phenomenon observed in remote high-altitude areas is the occurrence of gastrointestinal diseases attributed to environmental factors, particularly hypobaric hypoxia. As a pivotal factor, hypoxia has garnered increasing attention in both animal (primarily mice) and human populations. Previous research has shown that short-term exposure to hypoxic environments accumulates inflammatory cells in multiple organs. [7,8,20,24]. Notably, the small intestine serves as a vital barrier against bacterial translocation and endotoxin. It is well-established that intestinal mucosal integrity is compromised under hypobaric hypoxic conditions, with hypoxia identified as a significant risk factor for intestinal tissue injury [1,11,16,23,32]. Nevertheless, the specific mechanisms through which hypoxia disrupts the integrity of the Intestinal Mucosal Barrier (IMB) remain poorly understood.

In human populations, healthy climbers at high altitudes exhibit increased levels of various cytokines, including IL-6 and CRP, along with alterations in transport proteins and lipid metabolism within the intestinal mucosa [9,13,29]. Exposure to high altitudes can result in gastrointestinal erosion and ulceration. [22,27,30. The gastrointestinal stress response is largely attributed to digestive disorders induced by hypoxia, which adversely affects intestinal immune function, resulting in symptoms such as indigestion and abdominal distension [14].

Despite these findings in humans, little is known about the pathophysiological consequences of small intestinal tissue damage in rats. This knowledge gap has motivated us to further investigate gene expression changes in intestinal tissues under hypobaric hypoxia through RNA-Seq analysis.

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Materials and Methods

Experimental Animals

We randomly assigned twelve adult male Sprague-Dawley rats (SD) to three groups, each comprising four rats. These groups included normal rats (Z), rats exposed to hypobaric hypoxia for 2 weeks (W2Z), and rats exposed to chronic hypobaric hypoxia for 4 weeks (W4Z). Unfortunately, one rat from the W2Z group did not survive the hypobaric hypoxia exposure. All animals were housed under consistent conditions, with a temperature of 25±2°C, relative humidity of 50±10%, and a 12-hour light/dark cycle. The hypobaric hypoxia environment was simulated to mimic a 5,500-meter-high atmospheric condition using the FLYDWC50-1C hypobaric hypoxia cabin (Guizhou Fenglei Air Ordnance LTD, Guizhou, China). Standard rodent chow and water were provided ad libitum for each group. After an overnight fast, all rats were euthanized under 10% Pentobarbital Sodium anesthesia (0.4 ml/100g body weight, IP). Small intestinal tissue was collected under normobaric conditions, following the same procedures for rats subjected to hypobaric hypoxia for either 2 or 4 weeks. The small intestinal tissues were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Control rats (Z) were sacrificed under the same environmental conditions on the first day and processed identically to the other groups. All sample preparations were completed within one hour of collection.

The Small Intestinal Tissue RNA Quantification and Qualification

RNA degradation and contamination were assessed on 1% agarose gels. RNA purity was checked via the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured via a Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed via the RNA Nano 6000 Assay Kit from the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library Preparation for the Small Intestinal Transcriptome Sequencing

One microgram of RNA per sample served as the input material. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). Index codes were added to attribute sequences to each sample, following the manufacturer's recommendations. Total RNA underwent mRNA purification with poly-T oligo-attached magnetic beads, followed by fragmentation in NEBNext First-Strand Synthesis Reaction Buffer (5X) under elevated temperature. First-strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-).

The second-strand cDNA was generated using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3' ends of DNA fragments, NEBNext Adaptors with a hairpin loop structure were ligated to prepare for hybridization. Library fragments in the 250-300 bp range were selected via purification with the AMPure XP system (Beckman Coulter, Beverly, USA). Three microliters of USER Enzyme (NEB, USA) were used with size-selected, adaptor-ligated cDNA at 37°C for 15 minutes, followed by 5 minutes at 95°C before PCR. Subsequently, PCR was conducted with Phusion High-Fidelity DNA polymerase, universal PCR primers, and index (X) primers. The library quality was assessed using the Agilent Bioanalyzer 2100 system.

Clustering and Sequencing the Small Intestinal Gene

On a cBot Cluster Generation System the index-coded samples were clustered via TruSeq PE Cluster Kit v3-cBot-HS (Illumina) following the manufacturer's instructions. On an Illumina Hiseq platform, the library preparations were sequenced, and 125 bp/150 bp paired-end reads were generated.

Data Analysis and Quality Control

Raw data (raw reads) in fastq format were first processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapters, reads containing a ploy-N, and low-quality reads from the raw data. At the same time, the Q20, Q30, and GC content of the clean data were calculated. All the downstream analyses were based on high-quality, clean data.

Mapping Reads to the Reference Genome

The genome website directly obtained the reference genome and gene model annotation files. We constructed an index of the reference genome and aligned the paired-end clean reads to the reference genome using Hisat2 v 2.0.4. Hisat2, selected as the mapping tool, can generate splice junction databases based on the gene model annotation file, resulting in superior mapping outcomes compared to other non-splice mapping tools.

Quantification of the Small Intestinal Gene Expression Levels

HTSeq v 0.9.1 was used to count the read numbers mapped to each gene. An FPKM for each gene was calculated based on the length of the gene and the read counts mapped to the gene. FPKM (expected number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) considers the effect of sequencing depth and gene length for the read counts simultaneously and is currently the most commonly used method for estimating gene expression levels.

Differential Expression Analysis (DEGs)

Raw read counts of each sample were obtained using HTSeq v 0.9.1. Normalized gene expression data were acquired using the calcNormFactors function from the edgeR package and the voom function from the limma package. Differential expression analysis was performed using the limma package to compare the three groups, with filtering criteria requiring a corrected p-value less than 0.05 and a minimum absolute log fold change exceeding 2 in at least one of the three comparisons (W2Z versus Z, W4Z versus W2Z, and W4Z versus W2). The cluster profile packages were used for KEGG and Gene Ontology analysis of the DEGs, employing default parameters. Co-expression pathway analysis was conducted using the Cogena package.

Results

Different Small Intestine Transcritional Expression between Three Groups

We performed clustering analysis on the small intestinal tissues of the three groups based on Differentially Expressed Genes (DEGs). Notably, the results exhibited distinctions between acute hypobaric hypoxia (W2Z) and chronic hypobaric hypoxia (W4Z). A total of 266 DEGs were identified. The heatmap in Figure 1 illustrates the hierarchical clustering of these DEGs. It is evident that the expression patterns of DEGs among the three groups differed significantly. The W4Z group clus-

tered more closely with the control group, while the W2Z group showed marked differences.

We further conducted a Principal Component Analysis (PCA) using all the DEGs, as presented in Figure 2. This analysis revealed clear separation among the three groups, with small intestine samples within the same group clustering together. These results suggest the successful establishment of acute and chronic hypobaric hypoxia models. The horizontal principal component explained approximately 51.91% of the DEG variation, while the vertical principal accounted for 23.03% of the variation.

Gene Ontology Analysis and the KEGG Over-Representation between Three Groups

To elucidate the biological functions of the DEGs responding to acute and chronic hypobaric hypoxia, we conducted a Gene Ontology (GO) analysis and a KEGG over-representation test. The KEGG analysis, shown in Figure 3, highlighted the enrichment of the "Neuroactive ligand-receptor interaction" pathway in the hypobaric hypoxia environment. Additionally, several metabolic pathways, including "Retinol metabolism," "Steroid hormone biosynthesis," "Chemical carcinogenesis," and "Protein digestion and absorption," were enriched.

For GO analysis (Figure 4), in terms of Biological Processes (BP), we observed enrichment in nutrient transport, such as "Organic Anion Transport (OAT)" and "organic acid transport," as well as vasculature-related processes, including "angiogenesis," "regulation of angiogenesis," and "regulation of vasculature development." Furthermore, processes related to fatty acid metabolism, such as "fatty acid derivative metabolic process" and "unsaturated fatty acid," were enriched. In the Molecular Function (MF) category, transporter activities like "metal ion transmembrane transporter activity," "active transmembrane transporter activity," and "secondary active transmembrane transporter activity" were enriched. Finally, in the Cellular Components (CC), the DEGs were associated with the "cell part" and "plasma membrane."

Cogena Co-Expression Analysis between Three Groups

The co-expression analysis using Cogena revealed three distinct co-expression patterns, as depicted in Figure 5. Cogena clustered DEGs with similar functions together, providing a means to refine the understanding of DEGs and pathways. Most of the 431 DEGs were downregulated in the first cluster up to week 4. The second cluster, comprising 139 DEGs, exhibited downregulation at week 2 but upregulation at week 4. The third cluster, involving 283 DEGs, showed upregulation following hypobaric hypoxia.

Cogena pathway analysis was conducted for the three clusters using the Cogena Bioconductor package. The k-means clustering method and the three clusters for analysis were selected based on the Cogena manual. Notably, "Neuroactive ligand-receptor interaction" and "complement and coagulation cascades" pathways exhibited significant differences across all clusters. Interestingly, the "Neuroactive ligand-receptor interaction" pathway was exclusively enriched in the first cluster (Figure 6), while the "complement and coagulation cascades" were enriched in the second cluster but not in the others.

Discussion

In recent years, the significance of hypoxia in the pathogenesis of Gastrointestinal (GI) diseases and Intestinal Mucosal Barrier (IMB) impairment has gained increasing recognition. Several factors can contribute to intestinal mucosa injury, with hypoxia being a significant risk factor [1,2,11,16,23]. Hypoxia induces notable alterations in the small intestine, liver, and gut functioning, particularly in adult animals. The small intestine is crucial in the protective, restorative, and pathological responses to oxidative stress during hypoxia. Notably, carbohydrate metabolism is impaired at altitudes above 4300 meters, resulting in acute symptoms like anorexia, nausea, vomiting, weight loss, and malaise. Various physiological changes have been described at high altitudes, including increased basal glucose uptake, hyperinsulinemia, alterations in catecholamine concentrations, and elevated cortisol levels (Baranova et al. 2009). Hypoxia represents a pivotal feature of high-altitude environments, impacting overall health and disease through diverse effects on various organ functions [31,35]. With its roles in energy metabolism and immune function, the intestinal epithelium plays a vital part in the mucosal immune response and hosts numerous commensal bacteria [18,21].

Hypoxia influences basic metabolic processes and biological functions [34]. Additionally, it creates an acidic microenvironment due to carbonic anhydrase, which can be detrimental to individuals at high altitudes [6]. Hypoxic conditions also disrupt the balance of the gut microbiome, contributing to IMB [33]. Hypoxia has been associated with reduced secretory immunoglobulin A (sIgA) secretion in the small intestine [3,4]. Furthermore, hypoxia impacts small intestinal function, bacterial proliferation, bile secretion, and enterohepatic circulation, and can damage intestinal biological barriers [36]. This leads to the generation of high levels of Reactive Oxygen Species (ROS), contributing to oxidative stress and tissue injuries, including intestinal damage [15,25].

Histological examination (HE staining) has revealed pathological changes in small intestinal mucosa, possibly due to the overexpression of the TLR4/NF-κB pathway under acute hypobaric hypoxia [28]. These changes include higher lymphocyte infiltration in the lamina propria of intestinal epithelium and structural atrophy. Hypoxia is believed to modify the gut microbial imprint, leading to epithelial barrier dysfunction, potentially allowing the influx of endotoxins and activation of systemic inflammation [1]. In the intestines of Tibetans living at high altitudes, histology has revealed reduced and irregular intestinal villi (above 3650 meters) and damaged glandular epithelium. These changes appear to be closely associated with immunological responses [12].

Our findings emphasize the significance of complement and coagulation cascades in adapting to hypobaric hypoxia and suggest their potential utility in developing diagnostic and therapeutic biomarkers for gastrointestinal diseases induced by high-altitude hypobaric hypoxia. Our research strategy focused on common Differentially Expressed Genes (DEGs) and enriched pathways across various primary sites, enabling us to identify genes specific to this condition. We uncovered DEGs associated with the "neuroactive ligand-receptor interaction" pathway and the "complement and coagulation cascades." Our comprehensive analysis of rat small intestinal transcription sheds light on the crucial role of the complement system in maintaining physiological defense, cellular integrity, and homeostasis. Disruptions in complement regulators, which control the spontaneously activated complement cascade, can have repercussions on tissue health and lead to autoimmune diseases [5,26]. High-altitude natives also displayed down-regulation of two proteins,

complement component C3 (spot no: 11) and C4A (spot no: 18), in their plasma. This observation aligns with previous research and underscores the adaptive significance of complement components C3 and C4A in high-altitude natives.

In line with our findings, it appears that the mechanism of minor intestinal injury involves the regulation of the "neuroactive ligand-receptor interaction" pathway. As indicated by the DEGs, this pathway seems to be intricately involved in the response and adaptation to hypobaric hypoxia. Our study lays the foundational understanding of transcriptional changes in the rat small intestinal tissue exposed to acute and chronic hypobaric hypoxia. It suggests that both the "neuroactive ligand-receptor interaction" and "complement and coagulation cascades" pathways in the small intestine play pivotal roles in acclimatization to acute and chronic hypobaric hypoxia, potentially explaining mechanistic differences at high altitudes. Our insights were gleaned by comparing DEGs in the small intestine of rats exposed to hypobaric hypoxia for 1 to 2 weeks, providing insights into the molecular pathogenesis of gastrointestinal issues.

In summary, our study delves into the adaptive response of the small intestine to hypobaric hypoxia. Gene Ontology (GO) analysis highlights processes related to nitrogen compound metabolism, organic anion transport, angiogenesis, and regulation of vasculature development. Specifically, the "neuroactive ligand-receptor interaction" pathway and the "complement and coagulation cascades" were identified as major players in the small intestine's adaptation to a hypobaric hypoxic environment. This research contributes essential knowledge about the transcriptional mechanisms underlying small intestinal injuries at high altitudes and paves the way for subsequent gene validation and functional investigations.

Author Statements

Author Contributions

Formal analysis, Jiayu Xu. ; Investigation, Chengliang Yin.;Project administration, Kunlun He.; Software, D.T.; Supervision, Chengliang Yin., Jiayu Xu., Li Da., Liqiang Fu and Ming Lv.;Writing—original draft, Jiayu Xu.;Writing—review & editing, Ming Lv. Xue Bai and Chengliang Yin. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee of PLA General Hospital (2017-X13-05).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

This work has not been published anywhere else.

Conflicts of Interest

The authors have no potential conflict of interest to declare.

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