Quantitative analysis of the global proteome in lung from mice with blast injury

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Abstract

Background The lung is very easily injured by primary shock wave in recent military conflicts. While it is not very clear for its mechanisms. Protein markers can provide new ideas for diagnosis and treatment.

Methods A quantitative analysis of their global proteome was conducted in lung from mice with blast injury using LC-MS/MS. Protein annotation, unsupervised hierarchical clustering, functional classification, functional enrichment and cluster, and protein-protein interaction analyses were performed. Furthermore, western blotting was used to verify the differential protein.

Results A total of 6498 proteins were identified, of which 5520 proteins were quantified. The fold-change cutoff was set at 1.2; 132 proteins were upregulated, and 104 proteins were downregulated. The bioinformatics analysis indicated that the differentially expressed proteins were involved in the cholesterol metabolism, asthma, non-alcoholic fatty liver disease and so on. Remarkably, the processes related to the change of oxidative phosphorylation including the NADH dehydrogenase, Cytochrome C reductase, Cytochrome C oxidase and F-type ATPase were significantly upregulated, which were further verified by western blotting.

Conclusion These results confirmed that the oxidative phosphorylation is critical to blast-induced lung injury. LC/MS-based profiling presented candidate target/pathways that could be explored for future therapeutic development.

Background

The increased frequency of combat and terrorist operations throughout the world enhances the occurrence of blast injuries [1]. The target organs of blast waves are those that contain air or include structures with different specific weights (ear, lungs, intestine) [2]. Blast injuries are in general characterized with the absence of external injuries and with simultaneously present extensive lungs parenchymal damage [3, 4]. In our previous studies, we found that blast could induce lungs injury. The changes of pulmonary edema, inflammatory cell infiltration, and cell damage factor expression increased gradually with time, and reached the peak at 12–24 h after the outbreak. However, due to a lack of literature report and systematic comparative studies, there are limited understanding about the pathophysiology of blast-lung injury [5, 6]. Therefore, an urgent need still exists to understand the protein molecular changes underlying the impact of primary blast-induced lung injury.

The proteomic can reflect differences in metabolism and other properties between distinct states, and provides an actual measure of the biological impact of specific expression changes [7, 8]. It is a powerful approach used to understand complex biological systems and determine relationships between proteins, their function, and protein-protein interactions [9]. Mass spectrometry (MS)-based proteomics is a specific and sensitive tool to determine protein abundance in lung tissues and to predict biochemical responses. Elucidating the specifically activated proteins could provide therapeutically actionable pathways. [10, 11].

Therefore, we established an mouse model to simulate primary shock wave injury in this study. The proteomic including tandem mass tag (TMT) labeling, LC-MS/MS platform and advanced bioinformatics analysis were used to profile mouse lung suffering blast-induced injury. To the best of our knowledge, this is the first study to investigate the proteomic signature in in lung from mice with blast injury. The results would evaluate the knowledge on effect of blast and contribute to the diagnosis and treatment of blast-induced lung injury.

Methods

Animals

Male C57BL/6 mice weighing 18–22 g and aged 6–8 weeks were purchased from Liaoning Changsheng Bio-Technology Company Ltd. (Liaoning, China). All mice were allowed free access to food and water. Animal welfare and experimental design were approved by the Ethics Committee of the General Hospital of Northern Theater Command. Establishment of blast injury mice was conducted as previously described [12]. The blasting simulation device consists of four parts: air compression device, fixture, protection device, and data acquisition device. The bottom of the device is the air compression device, about 100 cm of steel pipe connected to the air pressure pump and power supply. The main device is above a 30 cm steel pipe, the top surface for the wire. The fixed protective cover contains the middle of the connection pressure sensor. The top of the main body and the lower device can be placed in the middle of different thicknesses of an aluminum film attached by screws.

Sample Collection

After 12 h of injury, mice were intraperitoneally anesthetized with 10% chloral hydrate (300 mg/kg). The abdominal cavity was opened, and lungs were harvested and frozen in liquid nitrogen for subsequent proteomics analysis and western blotting or placed in 4% paraformaldehyde for hematoxylin & eosin (H&E) staining.

Histological Examination

Paraffin sections were dewaxed in water, followed by hematoxylin staining for 5 min. After rinsing with water for 5 min, sections were incubated in 1% acetic acid ethanol for 30 s, stained with 0.5% eosin for 3 min and washed with distilled water for 30 s. After dehydration, sections were sealed with neutral gum and observed under a microscope.

Lc-ms/ms Analysis

After protein extraction, trypsin digest and TMT markers, the tryptic peptides were dissolved in 0.1% formic acid (solvent A) and directly loaded onto reversed-phase analytical column (Agilent 300Extend C18, 5 μ m, 4.6*250 mm). Liquid phase gradient setting: 0 ~ 20 min, 6%-20% B; 20 ~ 33 min, 20%-35% B; 33 ~ 37 min, 35%-80% B; 37 ~ 40 min, 80% B. Solvent A contained 0.1% formic acid in 2% acetonitrile; Solvent B contained 0.1% formic acid in 90% acetonitrile. All at a constant flow rate of 320 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 sec dynamic exclusion. Fixed first mass was set as 100 m/z. The distribution of peptide length identified by MS/MS was in accordance with the quality control requirements.

Database Search

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Trypsin/P was specified as cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and oxidation on Met was specified as variable modifications. FDR was adjusted to < 1% and minimum score for peptides was set > 40.

Bioinformatics Analysis

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database ([www. http://www.ebi.ac.uk/GOA/](http://www.ebi.ac.uk/GOA/)); Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathway. Wolfpsort, a subcellular localization predication soft was used to predict subcellular localization. All differentially expressed protein database accession or sequence were searched against the STRING database version 10.5 for protein-protein interactions.

Western Blot Assay

The protein samples were mixed with the corresponding sodium dodecyl sulfate (SDS) gel sample buffer, boiled for 5 min, and then transferred onto membranes for SDS-polyacrylamide gel electrophoresis. The membranes were blocked in phosphate-buffered saline with Tween 20 (PBST) containing 5% low-fat milk powder at room temperature for 1 h, washed three times with PBST and incubated with primary

antibodies (Sigma, St. Louis, MO, USA) overnight at 4°C. Subsequently, the blots were washed and incubated with secondary antibodies (Sigma, St. Louis, MO, USA). Antibody binding was detected by chemiluminescence staining using an ECL detection kit (Bio-Rad). The density of each band was quantified by densitometry using BandsScan 5.0 software.

Results

Blast exposure induced acute lung injury

According to the HE results, infiltration of inflammatory cells, and destruction of the alveolar architecture were observed in the the model group mice (Fig. 1). This result indicated that blast could induce acute lung injury, and the model could be used for further proteome study.

Quantitative Proteomic Analysis Of Differentially Expressed Protein

For global proteome analysis, the fold-change cutoff was set at 1.2 (models vs. controls); we identified 6498 proteins in lung tissues after blast exposure. Among these, there were 132 proteins were upregulated, and 104 proteins were downregulated as compared to sham control (Supplementary Table S1). The 236 differentially expressed proteins were divided into four quantitative categories according to blast vs. control ratios: $0 < \text{Ratio} < 1/1.3$, 48 proteins, $1/1.3 < \text{Ratio} < 1/1.2$, 56 proteins, $1.2 < \text{Ratio} < 1.3$, 61 proteins and $\text{Ratio} > 1.3$, 71 proteins (Fig. 2).

Protein Annotation And Functional Classification Of Differentially Expressed Protein

To understand the proteins identified and quantified, we annotated the functions and characteristics of these proteins from the aspects of Gene Ontology (GO), Protein domain, KEGG pathway, COG functional classification and sub-cellular structural localization (Supplementary Table S2). From biological process, cellular component, and molecular function results, the biological role of proteins were explained by GO secondary annotation classification. Among them, 84 proteins participate in cellular process; 85 proteins distribute in cell; and 89 proteins are binding proteins. Furthermore, we used software to localize, predict and classify differentially expressed proteins. Cytoplasm (27.27%) and mitochondria (18.94%) exist the maximum amount of differential protein. COG/KOG function classification were used to show the clusters of orthologous groups of proteins (Fig. 3).

Functional Enrichment Of Differentially Expressed Proteins

In order to find out whether the differentially expressed proteins had significant enrichment trends in some functional types, enrichment analysis of GO classification, KEGG pathway and protein domain were conducted. 20 of the most significant enrichment classifications are presented in the bubble chart. In the biological process category, the upregulated proteins were highly enriched in terms such as positive regulation of cytokine production, regulation of carbohydrate metabolic process, acute-phase response. The enrichment analysis of the cellular component category showed that proteins related to extracellular space, mitochondrial protein complex, mitochondrial membrane part and so on. According to the molecular function enrichment results, we found they are take part in NADH dehydrogenase (ubiquinone) activity, peptidase inhibitor activity, cyclic-nucleotide phosphodiesterase activity, serine-type endopeptidase inhibitor activity and so on (Fig. 4A-C). KEGG pathway enrichment, which is an information network that connects known intermolecular interactions, was further conducted to elucidate the biological functions of proteins. We found the changes of vitamin digestion and absorption, Asthma, oxidative phosphorylation and so on (Fig. 4D). In addition, protein domain, which refers to some components that occur repeatedly in different protein molecules were shown in Fig. 4E.

Analysis Of Functional Enrichment Cluster

Cluster analysis were performed to find the function correlations of the different proteins. Heat maps, which analyzed by clustering based on GO classification, KEGG pathway and protein domain enrichment, were shown in Fig. 5 (A. biological processes; B. cell composition; C. molecular function; D. KEGG pathway; E. Protein domains).

Changes Of Oxidative Phosphorylation Pathway

Based on the protein interaction network, we pay attention to oxidative phosphorylation pathway. The expression levels of NADH ubiquinone oxidoreductase subunits (NDUFV, NDUFA, NDUFB), Cyclooxygenase 2 inhibitors (COX2), Cytochrome c reductase Cytochrome c1 (cyt1), and F-type ATPase (OSCP) were upregulated according to KEGG pathway enrichment (Fig. 6A).

In order to further indentified the role of oxidative phosphorylation in blast-induced lung injury, western blot was used to detected the changes of related proteins. As shown by the western blot results (Fig. 6B), the blast at 12 hrs post injury led to a significant increase of complex I NDUFV1, NDUFA4, NDUFB3, NDUFB5, NDUFB6 subunit protein and COX2 in mice exposed to blast as compared to control.

Taken together, these results are in agreement with proteomics and bioinformatics data demonstrating oxidative phosphorylation involved with primary blast-induced lung injury

Discussion

In this study, we established a lung injury model using a self-developed experimental device and performed proteomics test at 12 h after injury according to our previous research. The acute lung injury caused by blast was verified by HE. The effects of blast on temporal protein changes were examined by global proteomes through comparing the proteins associated with the cellular components and functional pathways. Furthermore, oxidative phosphorylation proteins were detected by WB.

Blast injury is mainly caused by the primary shock wave. The internal organs present at the gas-liquid interface can be severely damaged [13]. Although proteomic analysis following low-intensity blast-induced mild traumatic brain injury has been reported, the critical players involved in blast-induced acute lung injury is not clear. The significance of this study is highlighted by an urgent need to identify mice responses to lung injury at the molecular level [14, 15]. Based on the proteomic profiles of mouse lung exposed to blast, we are exploring novel targets and candidate proteins linked to acute lung injury. Biological Process enrichment shown that the disruption of key canonical pathways. Enrichment pathways found these changes included multiple proteins associated with thermogenesis, complement and coagulation cascades, amino sugar metabolism, nucleotide sugar metabolism, oxidative phosphorylation and so on. These dysfunctions is related to the relevant enzyme activities. Thus, it is important to obtain the proteomic profiles of lung from mice suffered blast, these will provide a basis for the diagnosis and treatment.

Through in-depth mechanism research, our studies revealed the changes of oxidative phosphorylation including the NADH dehydrogenase, F-type ATPase, Cytochrome C reductase and Cytochrome C in the lung of blast-injury mice. Using Western blotting, we confirmed the change of several proteins, such as NDUFV1, NDUFA4, NDUF3, NDUF5, NDUF6, and COX. Oxidative phosphorylation occurs in the inner mitochondrial membrane [16]. It is the coupling reaction that utilizes substrates derived from glucose, fatty acids, and amino acids to produce ATP, which is a main source of organism energy.[17]. The enzymes of the oxidative phosphorylation consists of different protein complexes; The function is to carry out electron transfer, H transfer, oxygen utilization, and produce H₂O and ATP [18]. Complex I is NADH-Q reductase; Complex II is succinic acid-Q reductase; Complex III is cytochrome reductase; Complex IV is cytochrome oxidase; Complex V is ATP synthase [19]. In this study, NDUFV1, NDUFA4, NDUF3, NDUF5, NDUF6 all belong to NADH dehydrogenase. Ndufv gene relevant to mitochondrial respiration. It is reported that the NADH-dependent generation of extracellular superoxide was prevented by knockdown of NDUFV. [20]. Cyt1 belongs to cytochrome c reductase. Cytochrome c reductase exists as a dimer, each monomer contains two cytochrome b(b562-b566), a cytochrome c1 and a iron-sulfur protein. The founction is to catalyze electron transfer from coenzyme Q to cytochrome c [21]. COX belongs to cytochrome c oxidase. Cytochrome c oxidase is an enzyme at the end of the mitochondrial respiratory chain, it take part in the electron transport in the mitochondrial respiratory chain and be related to the production of reactive oxygen species [22]. For each pair of electrons transferred, four protons are simultaneously pumped from the mitochondrial matrix to the membrane gap [23]. ATP synthase is widely distributed in the inner membrane of mitochondria and participates in the ATP generation [24]. The electron transfer to proton pumping across the mitochondrial inner membrane to generate a

transmembrane electrochemical potential and interferes with energy metabolism [25]. Furthermore, it is reported that the alterations in oxidative phosphorylation can be related to the change of oxidative stress and inflammatory processes, which is consist with our finding in blast -injury mice [26].

Conclusions

Results of this study provide information on specific proteins and oxidative phosphorylation patyway changes in blast-induced lung injury. Proteomics for blast-induced lung injury research represents an exciting new approach that can greatly help to address the complex pathology of this condition. These insights offer new strategies for identification of potential biomarkers and provide candidate targets for novel therapeutics, for prognosis, and as surrogate outcome measures for new therapeutic strategies. In addition, our study identified many differentially expressed proteins have never before been associated with blast-induced lung injury, and further studies on these proteins are necessary. Thus, the data presented here will enable new research approaches to understand the function of proteins in blast-induced lung injury.

Abbreviations

LC-MS: Liquid chromatograph-mass spectrometer; TMT: Tandem mass tag; HE: hematoxylin eosin; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; SDS: Sodium dodecyl sulfate; PBST: Phosphate-buffered saline with Tween 20;

Declarations

Acknowledgments

Not applicable.

Authors' contributions

HX J, YL B and MX H designed the experiments. Y L wrote the manuscript. CC T XY S and L S performed the experiments. YE L, and PF C completed the statistical analysis. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animal welfare and experimental design were approved by the Ethics Committee of the General Hospital of Northern Theater Command.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Representative histopathological images of each group (A:control group; B:blast group. HE staining, scale bar = 50 µm).

Figure 2

Histogram of the number distribution (A) and volcano plot (B) of differentially expressed proteins.

Figure 3

Functional classification of differentially expressed proteins. A: GO secondary annotation classification; B: Localization and classification of subcellular structures; C: COG/KOG functional classification.

Figure 4

Bubble plot of the most significantly enriched in functional enrichment. The top 20 classifications with the most significant enrichment are presented in the bubble plot. A-C: GO enrichment (A. Biological process; B. Cellular component; C. Molecular function); D: KEGG pathway enrichment; E: Protein domain enrichment.

Figure 5

Functional cluster analysis of differentially expressed proteins. The abscissa axis of heatmap represents different fold-changes (Q1: $0 < \text{Ratio} < 1/1.3$, Q2: $1/1.3 < \text{Ratio} < 1/1.2$, Q3: $1.2 < \text{Ratio} < 1.3$, Q4: $\text{Ratio} > 1.3$).

A

B

Figure 6

KEGG pathway enrichment of differentially expressed proteins in oxidative phosphorylation pathway (A) and western blot results of NDUFV1, NDUF4, NDUF3, NDUF5, NDUF6 subunit protein and COX2 (B).

Supplementary Files

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