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Proteomics and Acetylation Omics Analysis of Endoplasmic Reticulum Stress-Mediated Fibrosis in Silicosis

Min-xia Zhu[1,*], Hong-Yang Shi[2,*], Song-Yu Gong[1], Zheng-Shui Xu[1], Jian-Tao Jiang[1,#] and Jian-Zhong Li [1,#]

[1]Department of Thoracic Surgery, the Second Affiliated Hospital of Xi'an Jiao Tong University, China

[2]Department of Respiratory and Critical Care Medicine, the Second Affiliated Hospital of Xi'an Jiao Tong University, China

[*]Equally contributing authors.

[#]**Corresponding author**: Jian-Zhong Li, Department of Thoracic Surgery, Second Affiliated Hospital of Xi'an Jiao Tong University, Xi'an, 157 Xi Wu Road, Xin Cheng District, Xi'an, Shanxi Province, China, E-mail: jianzhong-0520@163.com

Jian-Tao Jiang, Department of Thoracic Surgery, Second Affiliated Hospital of Xi'an Jiao Tong University, Xi'an, 157 Xi Wu Road, Xin Cheng District, Xi'an, Shanxi Province, China. E-mail: jiangjiantao2022@163.com

Abstract

Background: Rats treated with silicon dioxide (SiO_2) induced endoplasmic reticulum (ER) hyper-acetylation in pulmonary tissues and correlated with increased ER stress and lung tissue injury. Silicosis is a severe occupational disease caused by long-term inhalation of silica particles and is characterized by pulmonary fibrosis, dysfunction, and infection. Protein post-translational (PTM) modifications are involved in many physiological and pathological processes, with hyper-acetylation involved in autophagy, apoptosis, protein folding, and metabolism. In our silicosis rat model, resident ER protein acetylation interfered with correct protein folding and increased unfolded or misfolded proteins in the ER, consistent with increased abnormal

protein levels and stress in the ER.

Objectives: We investigated lung fibrosis mechanisms in silicosis-induced rats by analyzing proteomics and acetylation omics.

Methods and results: Excessive acetylation of protein disulfide isomerase (PDI) inactivated protein function. Also, ER stress abnormally activated three receptors and induced apoptosis in alveolar epithelial cell, the mesenchymal transition of smooth muscle, and the abnormal activation of inflammatory factors, which caused lung fibrosis.

Conclusions: PDI inactivation due to excessive acetylation caused abnormal protein maturation and accumulation leading to ER stress, and is a putative lung fibrosis mechanism in our silicosis-induced rat model.

Keywords: Fibrosis; Endoplasmic reticulum stress; Lysine acetylation; Post-translational modification; Protein disulfide isomerase

Introduction

Silicosis

Silicosis is a common occupational disease caused by exposure to free Silicon Dioxide (SiO_2) particles and is characterized by lung fibrosis and silicon nodule formation **[1].** These pathogenic processes occur over several steps: (1) Alveolar Macrophages (AMs) engulf silica and induce AM death; (2) Excessive inflammatory cytokine secretion causes lung inflammatory damage; and (3) Fibroblasts are transformed into myofibroblasts which cause excessive extracellular matrix deposition and eventual silicosis fibrosis **[2].** Silicosis is a severe public health global concern. Although preventive and monitoring measures are largely in place, disease prevalence and frequency remain high. According to the Chinese Ministry of Health, more than 23 million workers were exposed to silica particles, with a silicosis mortality rate of 23.1% from 1949 to 2008 **[3,4].** Although many studies have explored SiO_2 silicosis toxicity and identified complex biological and molecular mechanisms, no effective therapies exist to treat silicosis.

Post-translational modification (PTM) and acetylation

Protein PTM is a non-histone modification and a vital regulatory mechanism in the eukaryotic proteome which includes several different PTM types: phosphorylation, glycosylation, acetylation, methylation, and ubiquitylation. Lysine acetylation is a conserved PTM in prokaryotes and eukaryotes. In recent years, several

studies reported that non-histone protein acetylation had important roles in physiology and disease, including protein folding, autophagy, metabolism, and gene transcription [5]. Acetylation affects protein activity and protein stability by neutralizing positive charges in lysine [6]. Protein acetylation occurs in nuclear, cytoplasm, mitochondria, while N-acetylation occurs in the Endoplasmic Reticulum (ER). According to "ER acetyl-lysine proteomics", ER-resident and ER-transiting proteins undergo N-lysine acetylation in the ER. The main ER-resident proteins chaperone proteins and participating protein folding and PTMs. Additionally, ER acetylation regulates ER homeostasis by regulating autophagy and managing correctly folded polypeptides [7].

ER stress

The ER is an important organelle for the transportation, protein-folding, modification, and control of newly synthesized proteins. The ER maintains cellular protein homeostasis, with complex protein homeostasis networks involving disulfide catalysts, molecular chaperones, and regulators [8]. When unfolded or misfolded protein accumulation occurs in the ER, homeostasis is disturbed, stress is induced, and the Unfolded Protein Response (UPR) activated via genes involved in protein folding and anti-oxidative mechanisms. UPR signaling is initiated by three ER-membrane-associated proteins: (1) PKR-like eukaryotic initiation factor 2α kinase (PERK/eIf2α), (2) Inositol-Requiring Enzyme 1 (IRE1/Sxbp1), and (3) Activating Transcription Factor 6 (ATF6) [9]. (1) PERK is an eIF2α phosphorylated kinase - eIF2α phosphorylation is related to inflammation and several metabolic diseases, and activates expression of the transcription factor AFT4 and downstream targets such as pro-apoptosis factor CCAAT/enhancer-binding homologous (CHOP) and DNA damage proteins related to growth arrest (GADD34/PPP1R15a) [10]. (2) Activated IRE1α displays endonuclease activity, which transforms the mRNA encoding transcription factor XBP1 into activated XBP1. Activated IRE1α is oligomeric, auto-phosphorylated, and promotes mRNA and microRNA degradation [11]. (3) Under ER stress, ATF6 is transported to the Golgi apparatus and cleaved, then transported to the nucleus to promote expression of XBP-1 and genes related to ER protein degradation. Normally, these three pathways bind to the immunoglobulin heavy chain-Binding Protein (BiP) chaperone to maintain an inactive status. Active BiP (GRP 78) manages protein folding and maintains a balance between misfolded protein export and polypeptide import [12]. However, when ER stress is excessive, ER dysfunction occurs. Under chronic or prolonged ER stress, activated UPR joins different cellular signaling pathways implicated in fibrotic disorders in several organs, such as the kidney, liver, and lung [13-15]. Additionally, studies have shown that ER stress and UPR signaling in individual cell types are

linked to myofibroblast activation, macrophage polarization, and Epithelial Mesenchymal Transition (EMT) **[16].**

Protein disulfide isomerase (PDI)

PDIs function as enzymatic chaperones; they help reconstruct misfolded proteins in the ER and are involved in ER stress and the UPR. PDI family members contain thioredoxin-like catalytic **a** domain modules and non-catalytic **b** domains. The **a** domain contains CXXC active-site motifs, while **b** domains join substrates and provide interaction sites for co-factors **[17].** Disulfide bond formation in the ER depends on PDI and oxidoreductase. It is important for the ER to correctly fold transmembrane and secretory proteins. The nature of disulfide bond formation is redox reaction and the nature of thioglycolate. Disulfide bond formation depends on thiol groups on cysteine side chain residues in proteins. The local thiol group microenvironment influences PDI activity; positive charges adjacent to thiol groups make them more nucleophilic and provide stable electrostatic attractions for reactions with deprotonated thioates **[18].** PTMs have functional roles in highly conserved CXXC-flanking residues in mammalian PDIs; they affect oxidoreductase and thiol-reductase activity by limiting active site motion which is associated with disease **[19-29].** PDIA1 has important roles in protein folding, but a structurally abnormal protein contributes to delayed disulfide bond formation **[21].** Pathogenic PDIA3 features lead to the formation of protein aggregates, reduced enzymatic activity, and abnormal protein-protein interactions **[22].** For example, PDInitrosylation reduces PDI activity and is implicated in several neurodegenerative diseases, such as amyotrophic lateral sclerosis and Alzheimer's disease **[23,24].** In high glucose environments, the CXXC active site in PDI is succinylated and decreases PDI activity, abnormal proteins are accumulated in the ER, and the UPR is activated **[25].** Thus, PTMs in PDI may trigger ER stress and lead to disease; however, PDI acetylation mechanisms in silicosis remain unclear.

Materials and Methods

Study animals

Our three adult Sprague Dawley rat silicosis models and the other three control models complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication number 85-23, revised 1996). The study was authorized by the animal care and use committee of the Second affiliated Hospital of Xi'an Jiao Tong University.

The silicosis model

Silica particles (50 mg/ml) were sterilized at 120°C for 30 min and administered to rats by tracheal instillation under narcotism. Control rats were administered the same volume of sterilized saline. After one week, rats were humanely euthanized by injection with 10% KCL and the lungs of both groups fixed in 4% paraformaldehyde.

Histological scoring and Masson's staining

Right lower lung tissue was fixed in 4% neutral formalin solution for 48 h, followed by sequential dehydration, paraffin embedding, and serial 4-μm thick sections generated. Samples were hematoxylin & eosin (H&E) stained (BA4025, Baso Diagnostics Inc., China) to estimate lung tissue damage. Tissue sections were also stained using a Masson's staining protocol.

Total protein extraction

Lung tissue (from control and silicosis animals) was ground in liquid nitrogen and subjected to lysis buffer containing 100 mM NH_4HCO_3 (pH 8.0), 6 M urea, and 0.2% sodium dodecyl sulfate, followed by 5 min ultrasonication on ice. Lysates were centrifuged at 12000 × g for 15 min at 4°C and supernatants collected. Samples were reduced in 10 mM dithiothreitol for 1 h at 56°C, and alkylated with sufficient iodoacetamide for 1 h at room temperature in the dark. Then, samples were vortexed in four volumes of precooled acetone and incubated at -20°C for at least 2 h. Samples were recentrifuged and precipitates collected. After washing twice in cold acetone, pellets were dissolved in dissolution buffer, which contained 0.1 M triethylammonium bicarbonate (TEAB, pH 8.5) and 6 M urea.

Trypsin digestion

Supernatants, containing 10 mg protein, were digested in Trypsin Gold (Promega) at a 1:50 enzyme-to-substrate ratio. After 16 h digestion at 37°C, peptides were desalted on a C18 cartridge column to remove urea, and desalted peptides dried by vacuum centrifugation.

Tandem Mass Tag (TMT) labeling

To 120 μg protein, a protein solution was added up to 100 μl, then 3 μl trypsin (1 μg/μl) and 500 μl TEAB buffer (50 mM) were added, mixed, and incubated overnight at 37°C. Then, 100 μl 1% formic acid was added, mixed, incubated at room temperature, and centrifuged at 12000 × g for 5 min. The supernatant was passed through a C18 desalting column, washed three times in 1 ml cleaning solution (1% formic acid and 4% acetonitrile), washed two times in 0.4 ml eluent, and eluted. Then, 100 μl TEAB buffer (0.1 M) and 41 μl acetonitrile were

added to dissolve the TMT marks at the room temperature.

Immunoaffinity enrichment of lysine-acetylated peptides

After trypsin digestion, lyophilized peptides were resuspended in MOPS IPA buffer (50 mM MOPS, 10 mM KH_2PO_4, and 50 mM NaCl), while pH 7 was maintained with 1 M Tris, and centrifuged for 5 min at 12000 × *g*. Supernatants were mixed with anti-acetyl-lysine beads (CST#13416, Cell Signaling Technology) for 2.5 h, centrifuged for 30 s at 3000 × *g* at 4℃, the beads washed three times in MOPS IAP buffer, and recentrifuged for 30 s at 3000 × *g* at 4℃. Then, 15% TFA was added, samples incubated at room temperature for 10 min, and centrifuged three times for 2 min at 3000 × *g*. For liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis, peptides were desalted using peptide desalting spin columns (89852, Thermo Fisher). Take ammonia with a final concentration of 8 for reaction, marking with equal volume to obtain 9 samples, mixing, desalting and freeze drying.

LC-MS/MS analysis

For shotgun proteomics analyses, we used an EASY-nLCTM 1200 UHPLC system (Thermo Fisher) coupled to an Orbitrap Q Exactive HF-X MS operating in data-dependent acquisition mode. Peptides were separated on an analytical column (15 cm × 150 μm, 1.9 μm) using a 120 min linear gradient in 5%–100% eluent B (0.1% FA in 80% acn) and eluent A (0.1% FA in H_2O) at a 600 nl/min flow rate. The gradient was: 5%–10% B for 2 min; 10%–40% B for 105 min; 40%–50% B for 5 min; 50%–90% B for 3 min; and 90%–100% B for 5 min. A QExactive HF-x MS was operated in positive polarity mode with a spray voltage = 2.3 kV and capillary temperature = 320℃. Full MS scans, ranging from 350–1500 m/z, were acquired at a resolution of 60000 (at 200 m/z) with an automatic gain control (AGC) target value = $3x10^6$ and a maximum ion injection time = 20 ms. The 40 most abundant precursor ions from full MS scans were selected for fragmentation using higher energy collisional dissociation fragment analysis at a resolution of 15000 (200 m/z) with an AGC target value of 5e4, a maximum ion injection time = 80 ms, a normalized collision energy = 27%, an intensity threshold = 1.3e4, and a dynamic exclusion parameter = 30 s.

Data analysis

Corresponding databases were searched according to data generated by MS. According to the database (344833-X101SC19121138-Z02-mus+homo-uniprot.fasta (272302 sequences) identified the protein. MS quality was assessed by analyzing the mass tolerance of peptides, proteins, and parent ions. Then, protein quantitative

and differential analyses were conducted. Finally, differentially expressed proteins (DEPs) were annotated using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analyses. Based on the fact that protein molecular weights are altered when post-translationally modified, weights will be accurately determined by MS. Using protein quantitative and modification enrichment technology to quantitatively analyze PTMs and identify different modified sites and proteins.

Results

Lung fibrosis in the silicosis model

H&E staining of lung tissue in silicosis rats showed increased alveolar septal thickness and inflammatory cell infiltration when compared with control animals. More lung fibrosis was observed in silicosis than control rats. When compared with control animals, silicosis rats had increased collagen deposition. Lung fibrosis scoring in the lung tissue in silicosis rats, based on Szapiel's method, was higher when compared with control lungs.

Proteomics analysis

From the proteomics analysis of rat tissue, we identified 7685 proteins and determined DEPS between groups. A volcano map was used to show protein expression trends. In total, 242 proteins in lung tissue from silicosis-induced rats showed significant changes ($P<0.05$, log2 FC<-1 or log2 FC>1). When compared with control animals, silicosis-induced rats expressed 144 significantly up-regulated proteins while 98 proteins were significantly down-regulated. To further understand how DEPs regulated Biological Processes (BPs) related to silicosis pulmonary fibrosis, we used GO function enrichment analysis. In GO biological processes, DEPs were mainly involved in protein activation, protein synthesis, immune responses, and the regulation of external stimuli. These results were consistent with pathological processes caused by silicon particles during lung fibrosis.

DEP analysis of acetylation PTMs in the silicosis model

The lung tissues of model and control groups were examined using acetylation protein proteomics. We detected 4259 acetylation sites; 2951 were common sites in proteins, while 508 were different, including 201 and 307 significantly up- and down-regulated signatures, respectively. Moreover, 223 acetylated proteins were significantly different between groups. Acetylated protein heat map analysis showed that 101 acetylated proteins were up-regulated and 122 down-regulated ($P<0.05$, log2 FC<-1 or log2 FC >1) in the silicosis model. In peptide acetylation site analysis, most proteins had a single acetylation site, with no more than three sites. We

also analyzed different acetylation levels in the silicosis model using GO and KEGG function enrichment analyses. Based on GO annotations, proteins with acetylation differences were enriched in different BPs, including metabolic and biosynthetic pathways, immune responses, and cell proliferation. From KEGG pathway enrichment analyses, acetylated proteins were significantly related to biomass metabolism, amino acid synthesis, and protein degradation. Functional enrichment analyses of GO and KEGG terms showed that acetylated PTMs in model rats were significant for protein synthesis and degradation, metabolism, and immune responses. Subcellular organelle localization analysis showed that acetylated proteins were located in the nucleus, mitochondria, and cytoplasm, consistent with acetylation functions.

TMT and acetylomics

Further proteomic and acetylomic analyses showed that 126 proteins were differentially identified and 47 proteins were significantly up-regulated. GO enrichment annotation analysis of up-regulated proteins indicated that most were involved in protein synthesis and maturation processes, apoptosis, ER macrophagocytosis, extracellular matrix composition, and immune responses. These BPs were associated with silica-induced inflammation and fibrosis toxicology in the lung. From these protein analyses, PDI attracted our attention. Our results showed free differences in proteomics, while in acetylomics it was significantly upregulated in terms of acetylation. P4HB (proly4-hydroxylase, b polypeptide) belongs to the PDI family, is an ER resident, and is involved in protein folding and maturation. Abnormal protein folding causes aberrant protein formation and induces ER stress, which leads to pathological processes such as cell death, inflammatory responses, and EMT. These processes are consistent with tissue fibrosis.

Discussion

Silicosis is a common occupational disease. Long-term SiO_2 particle inhalation causes irreversible lung tissue damage and progressive lung function degeneration; however, no effective treatments are currently available. The toxic effects of SiO_2 particles on ER stress are known, but the underlying mechanisms require exploration. In this study, we analyzed acetylation omics in a silicosis-lung rat model and provided valuable disease and potential therapeutic insights. In cellular regulation networks, protein PTMs alters proteins and affects stability and activity. Because acetylated proteins occur at such low percentages in the proteome, their proteomic dissection is challenging. In our study, we identified three acetylation upregulated proteins P4HB, PDIA3, and

PPIB in the ER. P4HB (also known as PDIA1) is a PDI gene family member and is mainly localized to the ER. P4HB contains multiple acetylation sites which regulate protein activity by neutralizing positive charges in lysine. Thus, hyper-acetylation reduces protein activity and disturbs interactions between P4HB proteins. Weakened P4HB activity forms abnormal disulfide bonds and cross-linking between these bonds. Thus, chronic and prolonged misfolding exceeds ER self-regulation, leads to a disordered ER, and UPR signaling becomes activated via the three sensors: PERK, IRE1, and ATF6. The fibrogenic effects of ER stress are transduced by many lung cell types such as alveolar epithelial cells, fibroblasts, and alveolar macrophages.

Apoptosis

Fibrosis and silicon lung remodeling are related to alveolar epithelial cell apoptosis. Each of the three UPR sensors promotes different apoptosis outputs, which eventually lead to cell necrosis. The three UPRs regulate CHOP activation which is a central ER stress mediator. CHOP is involved in apoptotic pathways by upregulating pro-apoptotic gene activity and downregulating anti-apoptotic genes such as B-cell-lymphoma 2[26]. In a study shows that proapoptotic transcription factor CHOP, ATF6, and ATF4 stain in the AECII with fibrosis tissue under ER stress results in AECII apoptosis **[27].** Additionally, chronic ER stress increased the oligomerization state of IRE1α, which over-activates RNase domains in the cytoplasm and degrades various RNAs, including RNA precursors which inhibit apoptosis thereby promoting apoptosis **[28].** Under ER stress, IRE1α becomes auto-phosphorylated, and phosphorylated IRE1α regulates c-Jun NH2-terminal kinase (JNK) activity, which increases regulated IRE1-dependent mRNA and promotes cell death **[29].** Importantly, silica induces apoptosis in alveolar macrophages via ER stress **[30].**

Inflammation

ER stress pathways control several signal pathways and factors, such as JNK and NF-κB, which are involved in immunoregulation via protein and gene expression **[31].** The UPR, via IRE-1a, induces TNF receptor-associated factor2 (TRAF2) signaling to upregulate pro-inflammatory transcription factors such as NF-kB which is important in lung epithelial cell-activated silica-induced proinflammatory signaling **[32].** ER stress is associated with inflammasomes via IRE-1α. Inflammasomes activate cysteine protease-1, which initiates cell death pathways and cleaves pro-interleukin (IL)-b, pro-IL-18, and pro-IL-33 to generate mature IL-b, IL-18, and IL-33 molecules **[33].** Silica induces *in vitro* IL-1b via inflammasomes **[32,34].** Evidence also shows that ER stress induces inflammatory factors such as IL-b and IL-18, which have prominent roles in SiO_2-induced

inflammation and fibrosis [35]. ER stress alters the phenotypes of immune/inflammatory cells, especially macrophages. A previous study indicated that M2 type cells were dominant in pulmonary fibrosis, and that ER stress promoted M2 macrophage transformation.

EMT

ER stress also induces phenotypic metastasis in epithelial cells, and is required for fibroblast transformation to myofibroblasts in a fibrosis background [36]. Myofibroblasts secrete more collagens and extracellular matrix components which cause fibrosis [37]. A recent study demonstrated that silica-induced pulmonary fibrosis in mice was manifested by the extracellular deposition of collagen, fibronectin, and laminin [38]. A previous report also revealed that ER stress contributed to EMT in the Alveolar Epithelial Cell II (AECII) when exposed to SiO_2 particles [39]. Current evidence now shows that ER stress and UPR accelerate epithelial cell death, enhance inflammatory factor secretion, and promote cell differentiation induced-EMT in silica-induced models.

Autophagy

Autophagy is also involved in fibrosis development. Autophagy is a physiological process which helps regulate cell homeostasis; the process clears dysfunctional organelle, misfolded or unfolded proteins, and microorganism lysosomes to maintain ER homeostasis [40]. Autophagy also has key roles in acute and chronic lung diseases [41]. In our acetyl-proteomic analyses, the ER-resident proteins P4HB, PDIA3, and PPIB were acetylation upregulated thereby potentially inhibiting autophagy. Some studies suggested that long-term autophagy inhibition promoted renal fibrosis [42,43], hepatic fibrosis [44], heart failure [45], and neurodegeneration [46]. Specifically, our data indicated that autophagy inhibition may promote inflammation and fibrosis from silicosis [47].

We also observed that Fatty Acid Synthase (FASN) and lactic dehydrogenase (LDH) expression were both upregulated in silicosis rats. In a previous silicosis mouse model, LDH activity was upregulated, with airway protective agents reducing this activity [48]. FASN is involved in the *de novo* synthesis of fatty acids. Previous evidence indicated that lipid metabolism was implicated in inflammation and fibrosis [49,50], with pro-fibrotic Transformating Growth Factor-β(TGF-β) signaling required for FASN function [51]. However, acetylation mechanisms are unclear for LDH and FASN. However, many acetylated sites are enriched in energy metabolism pathways, therefore silicosis development may be metabolically related. More studies are required to explore this. Our study had some limitations. First, acetylation proteomics was based on lung tissue and did not identify

differences in cell types. Macrophages, fibroblasts, and AECII are involved in silicosis, but acetylation modification involved in regulating cell function and differentiation has been demonstrated in some studies. In future studies, we will clarify cell functions with respect to acetylation in SiO^2-induced rat models. Additionally, lung silicosis is a dynamic pathological process, from SiO^2 phagocytosis by macrophages to fibrosis. The molecular biology effects of silicosis should be observed at later stages to understand molecular mechanisms at different periods. Finally, our study was conducted without any quantitative analysis of acetylation activity, therefore we will address this in future studies.

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Author Contributions

Dr Jian-tao Jiang supervises and managers the study. Dr. Jian-zhong Li contributes to design the study and review the manuscript, Dr. Hong-yang Shi contributes to data curation. Min-xia Zhu contributed to transcript omics and proteomics analyses, and also wrote the original draft. Song-yu Gong contributes to validation. All authors read and approved the final manuscript. These authors contributed equally to this article.

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