Changes in Rat Urine Proteome Before and After Noise Exposure

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Abstract

This study established an acute noise exposure model, in which 10 rats were exposed daily to a 119 dB noise environment for 9 hours over 7 consecutive days, and proteomic analysis was performed on their urine samples. By comparing differential protein expression in urine before and after exposure, 219 differentially expressed proteins were identified, of which 30 are known to be associated with hearing impairment. Specifically, Gelsolin protein plays a crucial role in regulating the growth and stability of mechanosensory hair bundles in mammalian cochlear outer hair cells; deficiency in Lysosome-associated membrane glycoprotein 1 in mice leads to lysosomal membrane protein vacuolization and structural alterations in cochlear marginal cells, resulting in hearing loss; and loss of SLIT and NTRK-like family member 6 is associated with delayed synaptogenesis and hearing impairment in mice. These results indicate that exposure to high-decibel noise induces changes in urinary proteins, many of which have been reported to be associated with hearing loss.

Full Text

Changes in the Urinary Proteome of Rats Before and After Noise Exposure

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Abstract: This study established an acute noise exposure model in which 10 rats were exposed to a 119 dB noise environment for 9 hours daily over a period of 7 consecutive days. Proteomic analysis was performed on urine samples collected from the rats. A comparison of protein expression profiles before and after noise exposure identified 219 differentially expressed proteins, 30 of which are known to be associated with hearing loss. Notably, Gelsolin plays

a critical role in regulating the growth and stability of the mechanosensory hair bundles in mammalian cochlear outer hair cells. Deficiency of Lysosome-associated membrane glycoprotein 1 has been reported to cause vacuolization and structural alterations in lysosomal membrane proteins in cochlear marginal cells, leading to hearing loss in mice. Similarly, SLIT and NTRK-like family member 6 deficiency is linked to delayed synaptogenesis and auditory dysfunction in mice. These findings indicate that high-decibel noise exposure induces significant changes in urinary protein expression, many of which are associated with auditory damage as previously reported.

Keywords: urine; proteomics; damage to hearing; noise exposure; sensitivity of urine

A recent study published by the World Health Organization (WHO) revealed that up to 1 billion young people aged 12–35 worldwide are facing a severe risk of hearing loss, primarily due to prolonged and excessive exposure to high-volume music and other recreational sounds. As urbanization and industrialization accelerate in China, noise pollution has become increasingly prominent. Workers and visitors in noise-intensive environments such as military factories, airports, and entertainment venues face substantial risks of hearing damage, with noise-induced hearing loss emerging as a major cause of hearing disability that cannot be ignored.

In the diagnosis of hearing loss, temporary threshold shift (TTS) and permanent threshold shift (PTS) are common conditions. Although histological methods involving quantitative counting of synaptic markers are considered the gold standard, their complexity and invasiveness limit widespread clinical application.

Traditionally, research on hearing loss has relied on tissue sections, total RNA extraction, and total protein extraction from ear tissues or cells. However, an unprecedented research field—urine proteomics—is gradually emerging. This field demonstrates extraordinary potential for biomarker discovery and analysis, particularly in hearing loss, an area previously almost unexplored. The unique advantage of urine as a source of biomarkers lies in its lack of strict physiological homeostatic constraints, enabling more sensitive detection of subtle biochemical fluctuations within the body. Importantly, urine collection is both non-invasive and convenient, greatly enhancing its practicality and acceptability in biomarker research.

Numerous scientific studies have confirmed that urinary proteins can serve as biomarkers for various brain neurological diseases. Building upon this background, the present study aims to investigate the mechanisms of noise-induced hearing damage through the lens of urine proteomics. This innovative research approach not only promises to reveal novel mechanisms of noise-induced hearing damage but also provides unprecedented opportunities for developing new therapies for such hearing disorders.

Methods

2.1 Experimental Animals and Model Establishment

Twenty SPF-grade male Wistar rats (170–190 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed under standard conditions (room temperature 22$±$1 °C, humidity 65%–70%). Animal experiments were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University (approval number: CLS-EAW-2020-034). For model establishment, the 20 rats were randomly divided into two groups. One group of 10 rats was subjected to noise stimulation by exposure to 119 dB noise for 9 hours daily for 7 consecutive days, while the other 10 rats were maintained under normal conditions.

2.2 Urine Sample Processing

Prior to the experiment, the experimental group was placed in metabolic cages overnight for urine collection. Following noise stimulation, both the experimental and control groups were placed in metabolic cages overnight for urine collection. For urine sample processing, samples were first centrifuged at 12,000g for 30 minutes at 4°C. The components in each 15 mL urine sample were then precipitated with three to five volumes of ethanol at -20°C overnight. The following day, samples were centrifuged again at 12,000g, and the resulting protein pellets were dissolved in lysis buffer containing 8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L dithiothreitol. Protein concentrations in the supernatants were subsequently quantified using the Bradford assay.

For trypsin digestion, a total of 100 g of protein from each sample was loaded onto 10-kDa filtration devices and washed twice each with urea buffer and 25 mmol/L NH4HCO3 solution. Proteins were then reduced with 20 mmol/L dithiothreitol at 37°C for 1 hour, followed by alkylation with 50 mmol/L iodoacetamide (IAA) for 45 minutes in the dark. After additional washes with UA and NH4HCO3, samples were digested with trypsin (enzyme-to-protein ratio of 1:50) at 37°C overnight for approximately 14–16 hours. Digested peptides were desalted using Oasis HLB cartridges and lyophilized. For subsequent analysis, dried peptides were reconstituted in 0.1% formic acid and diluted to a concentration of 0.5 g/mL.

To construct a spectral library for data-independent acquisition (DIA) analysis, pooled samples (1–2 g each) were loaded onto equilibrated high-pH reversed-phase fractionation spin columns. A step gradient was created by adding volatile high-pH elution solutions containing eight different acetonitrile concentrations (5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 50% acetonitrile) to elute peptides across eight fractions. Eluted samples were dried by vacuum evaporation and resuspended in 20 L of 0.1% formic acid. Finally, 2 L from each fraction was used for liquid chromatography–data-dependent acquisition–tandem mass spectrometry (LC-DDA-MS/MS) analysis.

2.3 Data Analysis

Mass spectrometry data were acquired in DDA (data-dependent acquisition) mode for the ten fractions separated by reversed-phase chromatography. The DDA results were then imported into Proteome Discoverer software (version 2.1) for database searching. A DIA (data-independent acquisition) method was established based on the PD search results, with window width and number calculated according to m/z distribution density. Mass spectrometry data for individual peptide samples were acquired in DIA mode. Spectronaut X software was used to process and analyze the mass spectrometry data by importing raw DIA files from each sample for library searching. High-confidence proteins were defined as those with peptide q-values less than 0.01, and protein quantification was performed using the peak area of all fragment ions from secondary peptides.

2.4 Statistical Analysis

Each sample was analyzed in three technical replicates, and the resulting data were used for statistical analysis. Differential proteins were screened by comparing urinary proteins before and after noise exposure using the following criteria: fold change $ $1.5 or $ $0.67, and two-tailed paired t-test P-value < 0.05. In the random grouping analysis, the 20 samples were randomly divided into two groups, and the average number of differential proteins across all random combinations was calculated using the same screening criteria.

Results

3.1 Urinary Proteome Changes

LC-MS/MS tandem mass spectrometry analysis was performed on the 20 urinary protein samples collected before and after noise treatment. A total of 1,552 proteins were identified ($ $2 unique peptides, protein-level FDR $< 1\%$). Unsupervised clustering analysis of these data could distinguish urine samples collected before and after noise treatment. Figure 1 [Figure 1: see original paper] shows the detailed unsupervised clustering results.

3.2 Differential Protein Screening

Comparison of proteins before and after noise exposure identified 219 differential proteins (see appendix), while random grouping produced 15 differential proteins, as shown in Table 1 . The screening criteria for differential proteins were FC $ $1.5 or $ $0.67 and $P < 0.05$, with detailed information listed in Table 2 . Random matching by shuffling the groups yielded an average of 15 differential proteins, demonstrating that the differential protein results are reliable and not randomly generated.

Table 1 Random grouping

Table 2 Differential Proteins

Protein ID	Protein name
A0A0G2K5E1	Proline rich 4
F1LQQ8	Beta-glucuronidase (EC 3.2.1.31)
P01039	Cystatin-A
···	···(table continues)

3.3 Differential Protein Retrieval

For self-controlled before-and-after comparison, proteins were searched against the open-access PubMed database (https://pubmed.ncbi.nlm.nih.gov/), revealing 30 hearing-related proteins as shown in Table 3 . These proteins include Beta-glucuronidase, Lysosome-associated membrane glycoprotein 1, Receptor protein-tyrosine kinase, Biotinidase, L1 cell adhesion molecule, Plasminogen activator inhibitor 1, Alpha-mannosidase, Insulin-like growth factor-binding protein 3, Ciliary neurotrophic factor receptor subunit alpha, Low-density lipoprotein receptor-related protein 2, LIF receptor subunit alpha, Interleukin 1 receptor type 2, Cadherin 15, Ephrin B2, Arylsulfatase B, Gelsolin, protein-tyrosine-phosphatase, Ig-like domain-containing protein, Fatty acid-binding protein, CD44 antigen, Aquaporin-2, Transthyretin, Ig kappa chain C region, Choline transporter-like protein 2, Fibroblast growth factor receptor, Protein tyrosine kinase 7, Alpha-galactosidase, and SLIT and NTRK-like family member 6.

Notable findings among these proteins include: Biotinidase deficiency leads to hearing loss [4]. Lysosome-associated membrane glycoprotein 1 is expressed in the mouse cochlear organ of Corti [5], and alpha-mannosidase activity defects cause hearing impairment [7]. Insulin-like growth factor-binding protein 3 deficiency results in hearing and learning defects [8], while low-density lipoprotein receptor-related protein 2 serves as a biomarker in vestibular schwannoma-associated hearing loss. Interleukin 1 receptor type 2 is expressed in the inner ear, and its overexpression reduces noise-induced ribbon synapse damage and hearing loss [12]. Protein-tyrosine-phosphatase deficiency leads to deafness [18], and receptor protein-tyrosine kinase deficiency—specifically lack of collagen receptor DDR1—causes inner ear defects and hearing loss in mice [10]. Novel ILDR1 mutations in the Ig-like domain disrupt inner ear tricellular tight junctions, causing high-frequency hearing loss [19]. Protein tyrosine kinase 7 is associated with changes in tyrosine kinase B receptor levels 80 days after sound exposure and aging [26].

Additional hearing-related proteins identified include Arylsulfatase B variants, which cause hearing loss [16]. Gelsolin has complementary roles in regulating the growth and stability of mechanosensory hair bundles in mammalian cochlear outer hair cells [17]. FABP7 deficiency attenuates cochlear damage after noise exposure [20]. Deletion of the DDR1 gene in mice is associated with severe auditory function decline and major structural changes in the inner ear [10]. CD44 is expressed in the inner ear epithelium and is directly related to hearing

[21]. Aquaporin 2 shows early and specific expression patterns in the developing mouse inner ear, suggesting this aquaporin plays an important role in hearing development [22]. Transthyretin amyloidosis is associated with headache, hearing loss, and peripheral neuropathy [23]. SLC44A2 (solute carrier 44a2), also known as CTL2 (choline transporter-like protein 2), is expressed in many supporting cell types in the cochlea and is associated with hair cell survival and antibody-induced hearing loss [24]. Slitrk6-deficient animals show delayed synaptogenesis, and mutant mice exhibit auditory function defects reflecting human phenotypes [28].

In comparison with the 10 rats not exposed to noise, 46 differential proteins were identified, but random grouping produced 49 differential proteins, showing no significant difference in number. The possible reasons may be individual differences or environmental effects on the rats themselves. However, proteins directly related to hearing loss were still found among the 46 differential proteins.

Further analysis revealed additional hearing-related proteins. Frizzled 6 regulates the Wnt signaling pathway in mice, which modulates cochlear hair cell regeneration [29]. Proteoglycan 4 deletion mutations impair spatial coupling of pre- and postsynaptic elements, leading to hearing loss [30]. Apolipoprotein is directly associated with sensorineural hearing loss [38]. Calbindin regulates neuronal calcium concentrations during auditory conduction. Alpha-1 type XI collagen gene mutations cause Stickler syndrome type 2, which has been identified in mouse vitreous, cartilage, and cochlea, and is characterized by typical ocular abnormalities and auditory dysfunction [39]. Cochlear macrophages regulate cochlear inflammation and may have the potential to protect hearing function from damage, including acoustic overstimulation. Cochlear macrophage numbers increase 3–7 days after acoustic stimulation [32]. In the cochlear auditory epithelium, sensory hair cells and supporting cells are arranged in a checkerboard mosaic pattern that is conserved across species. Cell adhesion molecules nectin-1 and nectin-3 are required for this pattern formation. The checkerboard pattern is considered necessary for auditory function. Here, we demonstrate the importance of the checkerboard cellular pattern for survival and function of sensory hair cells in the cochlear auditory epithelium of nectin-3 knockout (KO) mice. Nectin-3 KO mice show progressive hearing loss associated with abnormal attachment and degeneration of hair cells through apoptosis. Apoptotic hair cell death results from disorganization of tight junctions between hair cells. Our study demonstrates that the checkerboard cellular pattern in the auditory epithelium provides a structural basis for ensuring cochlear hair cell survival and hearing function [34].

Fractalkine has regulatory functions in the nervous system and may affect auditory neuron function or protection [35]. Pharmacological treatment with annexin A1-derived peptide protects against cisplatin-induced hearing loss [36]. EphA2 regulates pendrin localization in the inner ear through interaction, affecting hearing function. Its mutation or dysfunction may cause hearing loss in patients with Pendred syndrome and enlarged vestibular aqueduct (EVA) [37].

This study employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology for the first time to search for clues of hearing loss from urine. A comprehensive and in-depth comparative analysis was conducted on the urinary proteomes of rats before and after noise exposure. The results revealed multiple significantly different proteins in the urinary proteomes, many of which were identified as directly related to hearing function. These hearing function-related differential proteins not only provide clues about the molecular mechanisms underlying hearing disorder development but also offer potential targets for future therapeutic development. This discovery deepens our understanding of the molecular mechanisms of noise-induced hearing damage and opens new research directions for early diagnosis and intervention of hearing disorders.

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