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Proteomic Analysis of Urine in a Rat E-Cigarette Model

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Date: 2023-01-13T00:00:00+00:00

Abstract

This study established a rat e-cigarette exposure model and collected urine samples at days 0, 3, 12, 15, and 17, representing pre-, mid-, and post-exposure stages, to investigate e-cigarettes through urinary proteomics. To eliminate the influence of individual differences, the experiment utilized a self-control design analyzing individual rats before and after exposure, while simultaneously establishing a control group to exclude variations caused by growth and development. The results demonstrated that under identical e-cigarette inhalation conditions, the differential proteins exhibited pronounced inter-individual variability. Among the differential proteins identified on day 3 of vaping, fetuin-B, a biomarker for COPD, and annexin A2, recognized as a marker for multiple tumors, were commonly detected in 5 out of 6 experimental rats in the before-and-after comparison. This study also revealed that odorant-binding proteins, expressed in the olfactory epithelium, appeared in the urinary proteome and were significantly upregulated, which may contribute to explaining olfactory adaptation. Furthermore, evidence of e-cigarette inhalation affecting the rat immune system, cardiovascular system, and respiratory system was identified in both the differential proteins and enriched signaling pathways, providing clues for further investigation into the mechanism of action of e-cigarettes on the human body.

Full Text

Urine Proteomic Analysis of a Rat E-cigarette Model

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Abstract: This study investigated the potential effects of e-cigarettes on the human body through urinary proteomics. We constructed a rat e-cigarette

model by exposing rats to e-cigarette aerosol for two weeks and collected urine samples before, during, and after the exposure period. To eliminate individual variation, we employed a before-after comparison for each individual rat, while a control group was established to exclude differences caused by normal growth and development. Our results revealed strong individual variation in the differential proteins produced by rats after e-cigarette exposure. Notably, among the differential proteins identified on day 3 of e-cigarette exposure, five out of six rats showed consistent identification of fetuin-B, a biomarker for COPD, and annexin A2, recognized as a multiple tumor marker. Surprisingly, odorant-binding proteins expressed in the olfactory epithelium were also detected and significantly upregulated in the urinary proteome, which may help explain olfactory adaptation. Furthermore, both the differential proteins and enriched signaling pathways provided evidence that e-cigarette exposure affects the immune, cardiovascular, and respiratory systems, offering clues for further investigation into the mechanisms of e-cigarette effects on the human body.

Keywords: urine; proteomics; e-cigarette model; odorant-binding protein

1. Introduction

1.1 E-cigarettes and Current Research Status

E-cigarettes consist primarily of four components: e-liquid, a heating system, a power source, and a filter. They generate aerosols with specific odors through heating and atomization for user inhalation. As of 2019, approximately 10 million people aged 15 and above in China used e-cigarettes, with the highest usage rate among the 15-24 age group. The majority (58.3%) of middle school e-cigarette users prefer fruit-flavored products, and previous studies have shown that these flavors may attract young people to try e-cigarettes. On May 1, 2022, China' s E-cigarette Management Regulations prohibited e-cigarettes with flavors other than tobacco.

Research by Pipe AL et al. has demonstrated that the heated chemical aerosols inhaled from e-cigarettes contain highly complex components, including nicotine, nitrosamines, carbonyl compounds, heavy metals, free radicals, reactive oxygen species, particulate matter, and "chemicals of emerging concern," further confirming the potential hazards of e-cigarette use. Studies have shown that e-cigarette use may increase the risk of pulmonary disease and cardiovascular disease, while also causing harm to the liver, urinary system, and immune system. Moreover, e-cigarette use not only endangers the user but may also harm fetuses exposed to e-cigarette aerosol during pregnancy. Ballbè M et al. detected low but non-negligible concentrations of e-cigarette-related analytes in umbilical cord blood and breast milk of pregnant women passively exposed to e-cigarette aerosol, while Aslaner DM et al. demonstrated that in utero exposure to secondhand e-cigarette vapor causes long-term pulmonary effects in offspring. Additionally, since the nicotine content in e-cigarette aerosol is comparable to or even higher than that in combustible cigarettes, its addictive potential cannot

be ignored.

1.2 Urine Biomarkers

Biomarkers are objective indicators that reflect normal or pathological biological processes and can predict, monitor, and diagnose multifactorial diseases at various stages. Compared with blood biomarkers, which are more widely used, the potential of urine biomarkers remains underdeveloped, particularly for early disease diagnosis and state prediction. Due to homeostatic regulation in blood, disease-induced changes in the blood proteome are metabolized and excreted, preventing early-stage diseases from showing significant changes. In contrast, urine is produced by glomerular filtration of plasma and is not subject to homeostatic regulation, making it highly sensitive to subtle early-stage disease changes. Furthermore, urine collection is non-invasive and readily accessible, making it an excellent source of biomarkers.

Urine biomarker detection has gained increasing attention from clinicians and researchers and has been applied to various diseases, including pulmonary fibrosis, colitis, and glioma. Studies have shown that urine biomarkers can classify diseases, such as predicting chronic kidney disease and distinguishing between benign and malignant ovarian tumors. Urine biomarkers can also detect whether tumors have been completely resected and predict recurrence risk, enabling timely adjustments to reduce recurrence. In pharmacology, urine biomarkers can monitor drug efficacy, such as predicting responses to rituximab treatment in adult systemic lupus erythematosus patients or demonstrating the superior effects of sacubitril-valsartan over valsartan in chronic heart failure treatment. In exercise physiology, urine biomarkers can reflect post-exercise changes in the urinary proteome, providing scientific evidence for athletes' training regimens. Recent studies have also identified urine proteomic biomarkers for neuropsychiatric diseases, including Parkinson' s disease, Alzheimer' s disease, depression, and autism.

However, no studies on e-cigarettes have been conducted in the field of urinary proteomics. Since the urinary proteome is susceptible to various factors such as diet, medication, and daily activities, a simple and controllable system is essential for accurate results. Animal models are highly suitable because their genetic and environmental factors can be controlled to minimize confounding influences. Therefore, we constructed an animal model to analyze the urinary proteome of rats exposed to e-cigarettes, with the experimental workflow shown in [Figure 1: see original paper].

[Figure 1: see original paper] Technical workflow for urinary proteomic analysis of the rat e-cigarette model

2.1 Establishment of the E-cigarette Animal Model

This study used 11 healthy male Wistar rats (8 weeks old, 180-200 g, SPF grade) purchased from Beijing Vital River Laboratory Animal Technology Co.,

Ltd. (Animal License No. SYXK(Beijing)2021-0011). All rats were housed in a standard environment (temperature (22$±$1)°C, humidity 65%-70%) and acclimatized for three days before the experiment. All procedures were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University.

The e-cigarette animal model was established as follows: 11 rats were randomly divided into an experimental group (n=6) and a control group (n=5). Control rats were maintained in a standard environment for 17 days. Experimental rats were exposed to e-cigarette aerosol once daily for 14 consecutive days. Each exposure involved atomizing one-third of a 3% nicotine pod (approximately 16 mg nicotine) and uniformly injecting the aerosol into two cages [36 cm (L) × 20 cm (W) × 28 cm (H)], with three rats per cage. The exposure lasted for 1 hour under conditions of adequate oxygen supply, after which the rats were returned to their home cages. Behavioral changes were observed throughout the experiment, and body weight was recorded every 5 days.

2.2 Urine Sample Collection

After three days of acclimatization, all rats were placed in metabolic cages to collect 12-hour urine samples. Additional 12-hour urine samples were collected on days 3, 6, 9, and 12 during e-cigarette exposure, and on days 1 (Day 15) and 3 (Day 17) after cessation of exposure. During urine collection, rats were deprived of food and water. All urine samples were stored at -80°C.

2.3 Urine Sample Processing

To observe the sensitivity of the urinary proteome and determine whether short-term e-cigarette exposure could induce detectable changes, we selected urine samples from day 0 (baseline), day 3 and day 12 during exposure, and days 1 and 3 post-exposure for detailed analysis.

Protein extraction and quantification: Urine samples from the five time points were centrifuged at 12,000×g for 40 min at 4°C, and the supernatant was transferred to new Eppendorf tubes. Three volumes of ice-cold absolute ethanol were added, mixed thoroughly, and precipitated overnight at -20°C. The mixture was then centrifuged at 12,000×g for 30 min at 4°C, and the supernatant was discarded. Protein pellets were resuspended in lysis buffer (containing 8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris), centrifuged at 12,000×g for 30 min at 4°C, and the supernatant was transferred to new tubes. Protein concentration was measured using the Bradford method.

Enzymatic digestion: 100 g of urinary protein was loaded onto a 10 kDa ultrafiltration membrane (Pall, Port Washington, NY, USA) placed in an Eppendorf tube, and 25 mmol/L NH_4HCO_3 solution was added to a final volume of 200 L. After adding 20 mM dithiothreitol (DTT, Sigma) and vortexing, the sample was heated at 97°C for 5 min in a thermomixer and cooled to room

temperature. Subsequently, 50 mM iodoacetamide (IAA, Sigma) was added, mixed, and incubated in the dark at room temperature for 40 min.

Membrane washing and digestion: (1) The membrane was washed twice by centrifugation at 14,000×g for 5 min at 18°C with 200 L UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5). (2) The processed sample was loaded and centrifuged at 14,000×g for 40 min at 18°C. (3) The membrane was washed twice with 200 L UA solution at 14,000×g for 40 min at 18°C. (4) The membrane was washed twice with 25 mmol/L NH_4HCO_3 solution at 14,000×g for 40 min at 18°C. (5) Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio, and digestion was performed overnight at 37°C. The following day, peptides were collected by centrifugation at 13,000×g for 30 min at 4°C, desalted using HLB columns (Waters, Milford, MA), dried in a vacuum concentrator, and stored at -80°C.

2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

Digested samples were dissolved in 0.1% formic acid, and peptide concentration was quantified using a BCA assay kit and diluted to 0.5 g/ L. A pooled peptide sample was prepared by mixing 4 L from each sample and fractionated using a high-pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific) according to the manufacturer' s instructions. Ten fractions were collected by centrifugation, dried, and reconstituted in 0.1% formic acid. iRT reagent (Biognosys, Switzerland) was added at a 10:1 sample-to-iRT ratio to calibrate peptide retention times. For analysis, 1 g of peptides from each sample was analyzed using an EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA) coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

For spectral library generation, the 10 fractions were analyzed in Data Dependent Acquisition (DDA) mode with high-sensitivity settings. Full MS scans were acquired at a resolution of 60,000 over the 350-1500 m/z range. Individual samples were analyzed in Data Independent Acquisition (DIA) mode using a 36-window DIA method. A pooled peptide sample was analyzed by DIA after every 10 samples as a quality control.

2.5 Database Searching and Label-free DIA Quantification

Raw data files from LC-MS/MS were imported into Proteome Discoverer (version 2.1, Thermo Scientific) and searched against the Swiss-Prot rat database (released May 2019, containing 8,086 sequences) with iRT sequences appended. Search results were then processed and analyzed using Spectronaut Pulsar (Biognosys AG, Switzerland). Peptide abundance was calculated by summing the peak areas of respective fragment ions in MS2, and protein intensity was determined by summing the abundances of its constituent peptides.

2.6 Data Analysis

Each sample was analyzed in two technical replicates, and average values were used for statistical analysis. This study employed before-after comparisons for experimental group samples at different time points, with a control group to exclude developmental effects. Differential proteins were identified using the following criteria: fold change (FC) $ $1.5 or $ $0.67, and two-tailed unpaired t-test P-value <0.01. Functional enrichment analysis of differential proteins was performed using the Wukong platform (https://www.omicsolution.org/wkomic/main/), UniProt (https://www.uniprot.org/), and DAVID database (https://david.ncifcrf.gov/). Published literature was searched in PubMed (https://pubmed.ncbi.nlm.nih.gov) for functional annotation of differential proteins.

3.1 Characteristics of the Rat E-cigarette Model

Throughout the modeling period, we observed behavioral changes in the rats. Control rats exhibited normal activity and feeding/drinking behaviors, whereas experimental rats showed significantly increased water consumption. Body weight was recorded every 5 days, and we observed notably increased individual variation in body weight among experimental rats compared to controls [Figure 2: see original paper].

[Figure 2: see original paper] Body weight changes in the rat e-cigarette model

3.2 Analysis of Urinary Proteome Changes in the Rat E-cigarette Model

(1) Urinary protein identification: Following establishment of the rat e-cigarette model, 55 urinary protein samples were analyzed by LC-MS/MS, resulting in the identification of 1,093 proteins (with $ $2 unique peptides and protein-level FDR <1%).

(2) Individual rat urinary proteome analysis during e-cigarette exposure: To investigate consistency among the six experimental rats, we performed before-after urinary proteomic analysis for each rat individually, comparing different time points to day 0. Differential proteins were screened using the criteria: FC $ $1.5 or $ $0.67, and two-tailed unpaired t-test P<0.05. The results are summarized in .

Differential protein expression changes in individual rats during e-cigarette exposure

To visually assess the degree of consistency among the six experimental rats, we generated Venn diagrams of differential proteins identified at days 3, 12, 15, and 17 compared to day 0 [Figure 3: see original paper]. The results revealed that most differential proteins were unique to individual rats, indicating strong individual variation in e-cigarette effects.

[Figure 3: see original paper] Venn diagram of differential proteins from before-after comparisons in six experimental rats

To validate associations among differential proteins at different time points, we compared days 3, 12, 15, and 17 to day 0 for each rat and generated a Venn diagram of proteins identified in five or more rats [Figure 4: see original paper]. Neural cadherin was identified in five or more rats at all four time points and consistently showed downregulation. Additionally, six differential proteins were identified in five or more rats at three time points, representing relatively common changes. The trends of shared differential proteins among the six experimental rats across different days are shown in .

[Figure 4: see original paper] Venn diagram of shared differential proteins among six experimental rats at different time points

Trends of shared differential proteins among six experimental rats across different days

When analyzing before-after comparisons of day 3 versus day 0 in the six experimental rats, we identified 2 proteins common to all six rats and 16 proteins common to five rats. After excluding proteins that also appeared in control group comparisons, we obtained experiment-specific shared differential proteins (details in Table S1). Notably, fetuin-B (FETUB) was identified in five rats, all showing significant downregulation, and remained significantly different on days 12 and 15. Studies have shown that fetuin-B is a biomarker for chronic obstructive pulmonary disease (COPD), demonstrating the sensitivity of urinary proteomics. Intriguingly, we also observed odorant-binding proteins (OBPs), including OBP1F and OBP2A, among the day 3 differential proteins. OBP1F is primarily expressed in rat nasal glands, while OBP2A is transcribed in the nose of both humans and rats, suggesting that inhaled odors can leave traces in the urinary proteome. Although the physiological roles of OBPs remain unclear, urinary proteomics may contribute to elucidating their mechanisms. Furthermore, the presence of OBPs in urine may provide insights into olfactory adaptation. Annexin A2, widely recognized as a multiple tumor marker, was also identified. Additionally, László ZI et al. have shown that neural cadherin is one of the most important cell adhesion molecules in brain development, playing crucial roles in neuronal formation, proliferation, differentiation, migration, axon guidance, synaptogenesis, and synaptic maintenance.

Similar analysis of day 12 versus day 0 comparisons identified 9 proteins common to all six rats and 18 proteins common to five rats (experiment-specific proteins in Table S2). OBP2B showed consistent upregulation among the six rats. Unlike OBP2A, OBP2B is primarily expressed in reproductive organs and weakly expressed in respiratory organs such as the nose and lungs. Studies indicate that desmocollin 3 is essential for cell adhesion and desmosome formation and may enhance angiogenesis and nasopharyngeal carcinoma metastasis, serving as a biomarker for cancers such as non-small cell lung cancer. Annexin A5 may influence tumor development, pulmonary fibrosis, and lung injury, and is used

as a biomarker in tumor and asthma research, potentially promoting laryngeal and nasopharyngeal carcinoma development. We also identified gelsolin, an important cellular target of nicotine metabolite cotinine, through which nicotine may affect fundamental processes of tumor transformation and metastasis such as migration and apoptosis. Heat shock proteins have been reported as major markers affected by cigarette smoke, participating in signaling pathways related to cell cycle, cell death, and inflammation.

Comparison of day 15 versus day 0 identified 7 proteins common to all six rats and 45 proteins common to five rats (experiment-specific proteins in Table S3). Ponmanickam P et al. have shown that alpha-2u globulin may serve as a carrier for hydrophobic odors from preputial glands, which play an important role in pheromone-mediated olfactory signaling in rats, suggesting that alpha-2u globulin likely participates in olfactory signal transmission.

Finally, analysis of day 17 versus day 0 comparisons identified 7 proteins common to all six rats and 42 proteins common to five rats (experiment-specific proteins in Table S4).

(3) Functional analysis of differential proteins from before-after comparisons: To explore the functions of these differential proteins, we performed pathway enrichment analysis using the DAVID database on proteins identified in five or more experimental rats (details in Table S5). Thirty-two biological processes were enriched by differential proteins from two time points.

We also conducted signaling pathway analysis on proteins identified in five or more experimental rats . Two pathways were enriched at two time points: legionellosis and ferroptosis. The lung injury caused by legionellosis may share mechanisms with e-cigarette effects. M. Yoshida et al. demonstrated that cigarette smoke can induce ferroptosis in epithelial cells, a pathway involved in COPD pathogenesis. Additionally, we enriched many pathways related to respiratory diseases, including the Apelin signaling pathway, folate biosynthesis pathway, and arachidonic acid metabolism. Apelin is an endogenous ligand for the G protein-coupled receptor APJ, and the Apelin/APJ pathway is closely associated with respiratory diseases, representing a potential therapeutic target. Stanisławska-Sachadyn A et al. showed that serum folate concentrations are higher in smokers than in healthy controls, suggesting that folate synthesis may be associated with increased lung cancer risk. Among the 23 enriched pathways, multiple were closely related to the immune system, and two were directly associated with cardiomyopathy and atherosclerosis, suggesting that e-cigarette exposure may affect the immune and cardiovascular systems. Importantly, we also enriched two pathways related to chemical carcinogenesis, including chemical carcinogenesis-DNA adducts and chemical carcinogenesis-reactive oxygen species, which may validate previous findings that e-cigarette aerosol contains carcinogenic chemicals.

Signaling pathways enriched by shared differential proteins from five or more experimental rats during e-cigarette exposure

4. Conclusion

This study constructed a rat e-cigarette model and employed individual before-after comparisons to investigate urinary proteome changes following e-cigarette exposure. The results demonstrated strong individual variation in differential proteins produced by rats under identical exposure conditions. On day 3 of e-cigarette exposure, we identified COPD biomarker fetuin-B and multiple tumor marker annexin A2 among the differential proteins, highlighting the sensitivity of urinary proteomics. We also detected significantly upregulated odorant-binding proteins expressed in the olfactory epithelium at multiple time points, which may provide clues for understanding olfactory adaptation, though the mechanism by which these proteins appear in urine remains to be elucidated. Both the differential proteins and enriched signaling pathways provided evidence of e-cigarette effects on the immune, cardiovascular, and respiratory systems, offering valuable clues for further investigation into the mechanisms of e-cigarette effects on human health.

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Author Contributions: Youhe Gao: conceptualization, methodology design, final manuscript revision; Yuqing Liu: experimentation, data acquisition, analysis, manuscript drafting; Ziyun Shen: experimentation; Chenyang Zhao: data analysis.

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