

## Urinary Proteomic Alterations in Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)

**Authors:** Meng Wenshu, Gao Youhe, Gao Youhe

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### Abstract

Hypertension is a multifactorial complex disease associated with genetic and environmental factors that has become one of the most serious public health challenges. Despite progress in blood pressure reduction, the diagnosis and treatment of hypertension remain limited due to insufficient investigation of relevant molecular mechanisms. Urine proteomics technology holds potential to provide novel research strategies for complex diseases involving multiple biological pathways and organs. In this study, we utilized the SHRSP rat model to simulate hypertension progression, collecting urine samples at months 1, 4, 8, 10, 12, and 14, and analyzed dynamic changes in the urine proteome using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Considering that disease progression may vary among individuals, we adopted a within-rat longitudinal comparison approach to screen differential proteins and performed functional annotation analysis to gain deeper insights into the pathological mechanisms involved in hypertension progression. Throughout disease progression, we identified enrichment of several critical pathways in urine, including renin-angiotensin signaling response to oxidative stress, aldosterone signaling in epithelial cells, renin-angiotensin signaling, IL-12 signaling and production in macrophages, apelin adipocyte signaling pathway, calcium transport I, STAT3 pathway, and glucocorticoid receptor signaling. These pathways have been previously reported to be associated with the pathophysiological mechanisms of hypertension and may offer novel clues for antihypertensive drug target research. This study demonstrates that urine proteomics can reflect pathological processes associated with hypertension, suggesting that this approach may be utilized to investigate hypertension mechanisms, identify new drug targets, and potentially provide strategies for personalized antihypertensive therapy with optimal efficacy.

## Full Text

### Study on Urinary Proteomic Changes in Stroke-Prone Spontaneously Hypertensive (SHRSP) Rats

Wenshu Meng, Youhe Gao\*

Beijing Key Laboratory of Gene Engineering Drug and Biotechnology, College of Life Sciences, Beijing Normal University, Beijing, 100875, China

## Abstract

Hypertension is a multifactorial complex disease influenced by genetic and environmental factors, representing one of the most serious public health challenges worldwide. Despite advances in blood pressure management, diagnosis and treatment remain limited by insufficient understanding of relevant molecular mechanisms. Urinary proteomics offers promising new research strategies for complex diseases involving multiple biological pathways and organ systems. In this study, we employed the stroke-prone spontaneously hypertensive rat (SHRSP) model to simulate hypertension progression, collecting urine samples at 1, 4, 8, 10, 12, and 14 months of age and analyzing dynamic urinary proteome changes using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Recognizing that disease progression rates may vary among individuals, we adopted a longitudinal within-subject design to screen for differential proteins, followed by functional annotation analysis to elucidate pathological mechanisms involved in hypertension progression with greater precision. Throughout disease development, we identified enrichment of several critical pathways in urine, including renin-angiotensin signaling in response to oxidative stress, aldosterone signaling in epithelial cells, renin-angiotensin signaling, IL-12 signaling and production in macrophages, apelin adipocyte signaling pathway, calcium transport I, STAT3 pathway, and glucocorticoid receptor signaling. These pathways have been previously implicated in hypertension pathophysiology and may provide novel clues for antihypertensive drug target discovery. Our findings demonstrate that urinary proteomics can reflect pathological processes associated with hypertension, suggesting that this approach may be valuable for investigating hypertension mechanisms, identifying new therapeutic targets, and potentially guiding personalized antihypertensive treatment strategies for optimal efficacy.

**Keywords:** Hypertension; Urine; Proteomics; SHRSP rats

## Introduction

Hypertension affects over 1.2 billion people globally, constituting one of the most severe public health challenges. As a multifactorial complex disease involving both genetic and environmental factors, hypertension contributes to 8.5 million deaths annually from stroke, ischemic heart disease, other vascular diseases, and kidney disease. The heavy burden of cardiovascular and cerebrovascular diseases on patients, families, and society has made the development of accu-

rate, efficient early diagnosis and treatment strategies to effectively slow or even halt disease progression one of the top ten scientific questions for human social development in 2021. Over the past two decades, no new drugs specifically for hypertension treatment have been developed. Most patients require two or more medications to control blood pressure alongside statins to reduce risk factors. Many clinicians add drugs sequentially without specific rationale, yielding limited benefits while generating unnecessary costs and side effects. Despite the availability of effective and safe antihypertensive agents, hypertension and associated risk factors remain uncontrolled in most patients. While drug responses are similar across populations, individual responses vary significantly, making it difficult to select the most effective antihypertensive regimen for each patient. Tools to predict drug responses could guide clinicians in medication selection and individualized therapy. The lack of molecular mechanism research limits hypertension diagnosis and treatment, necessitating novel strategies to reduce its public health impact.

Three primary mechanisms are known to elevate blood pressure in essential hypertension: volume status, the renin-angiotensin system (RAS), and the sympathetic nervous system (SNS). Most available drugs—including diuretics, ACE inhibitors (ACEIs), angiotensin receptor blockers (ARBs), direct renin inhibitors (DRIs), beta-blockers, alpha-blockers, and central alpha-agonists—target these pathways. Calcium channel blockers (CCBs) are FDA-approved for blood pressure reduction either alone or in combination with other agents. Despite various drug combinations, over 40% of patients have uncontrolled blood pressure. Several potential therapeutic targets have been identified, including Toll-like receptor 4 (a key regulator of angiotensin II-induced hypertension), protease-activated receptor 2, chemerin, apelin receptor, transient receptor potential melastatin, urotensin-II, and Tie2 receptor. While extensive research has sought to elucidate hypertension pathogenesis and develop new therapeutic strategies, more effective treatment options for blood pressure control are still needed.

Increasingly, omics technologies are being used to reveal hypertension pathophysiology, predict diagnosis and organ damage, forecast treatment responses, and monitor therapeutic efficacy. Although rapidly advancing proteomics technologies have been applied to clinical and translational research in various diseases, hypertension proteomics studies remain limited. Recent examples demonstrate the potential of proteomics in hypertension research. Matafora et al. conducted urinary proteomics studies in hypertensive patients to identify urine proteins associated with hypertension and salt sensitivity pathogenesis, finding that urinary renin 1 may predict salt-sensitive phenotypes. Kuznetsova et al. developed a peptide panel characteristic of early diastolic dysfunction in hypertensive patients using urinary proteomics; these peptides were also differentially expressed in overt heart failure patients versus controls, indicating that molecular processes involved in early organ damage stages persist in advanced disease. Pena et al. performed plasma proteomics in hypertensive and diabetic patients, identifying biomarker signatures for proteinuria state transitions that may fa-

facilitate early recognition of hypertension-related kidney complications. Gajjala et al. compared plasma proteomes between hypertensive patients and controls, discovering 27 differentially expressed molecules with known or potential roles in blood pressure regulation and cardiovascular disease.

Urine serves as an excellent source of disease biomarkers because it is not regulated by homeostatic systems and contains rich, early, and comprehensive physiological changes. Urinary proteomics has recently revealed changes related to brain or nervous system developmental disorders beyond the renal system in Alzheimer's disease, autism, depression, gliomas, pediatric medulloblastoma, and Parkinson's disease. Studies have shown that urinary proteomics can reflect pathophysiological changes in coronary artery disease. Zimmerli et al. compared urinary peptides between coronary artery disease patients and controls, identifying a peptide set reflecting coronary features, demonstrating that urinary proteomics can reliably identify coronary artery disease patients and potentially monitor therapeutic intervention effects. Dawson et al. explored urinary proteomes in stroke patients versus controls, developing a biomarker classifier model for stroke diagnosis. Wu et al. conducted urinary proteomics in carotid artery stenosis (CAS) patients and healthy controls, identifying novel potential urinary biomarkers for non-invasive early screening and risk stratification of CAS. These results suggest that urinary proteomics holds promise for providing new mechanistic insights into cardiovascular and cerebrovascular diseases involving multiple biological pathways and organ systems.

The stroke-prone spontaneously hypertensive rat (SHRSP) is an animal model that spontaneously develops complex cerebrovascular lesions. Developed from spontaneously hypertensive rats (SHR) through selective breeding and successive inbreeding, SHRSP represents a unique genetic model for both hypertension and stroke. SHRSP rats exhibit features similar to human intracranial hemorrhage, atherosclerotic thromboembolic infarction, and lacunar infarction. In this study, we used the SHRSP rat model to simulate stroke-prone hypertension, collecting urine at 1, 4, 8, 10, 12, and 14 months for data-dependent label-free quantitative proteomics analysis. Recognizing that disease progression may vary among individual rats, we employed a within-subject comparison approach to screen for differential proteins and performed biological pathway annotation analysis to better understand pathological mechanisms during disease progression, providing new clues for understanding biological processes in cardiovascular and cerebrovascular diseases and identifying novel drug targets. Additionally, this approach may enable personalized therapy selection for hypertensive patients through urinary proteome-based pathway analysis. The study workflow is illustrated in Figure 1.

## Methods

### Animal Models

Eighteen 4-week-old male SHRSP rats and six male Wistar rats weighing 140-160g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed under standard conditions (room temperature  $22\pm1^{\circ}\text{C}$ , humidity 65%-70%). Animal experiments were approved by the Ethics Committee of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Approval No.: ACUC-A02-2014-007).

### Urine Sample Collection and Processing

After 3 days of acclimatization in metabolic cages, urine was collected from each rat at 1, 4, 8, 10, 12, and 14 months of age. Rats were placed in metabolic cages from 8 AM to 6 PM for urine collection. Collected urine was centrifuged at 12,000g for 40 minutes, and the supernatant was precipitated overnight with 3 volumes of ethanol, followed by centrifugation at 12,000g for 30 minutes. Protein pellets were resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, and 50 mmol/L Tris). Protein concentration was measured using the Bradford method. Urinary protein digestion was performed using the filter-aided sample preparation (FASP) method. Urinary proteins were loaded onto 10 kDa ultrafiltration tubes (PALL), washed twice with UA (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) and 25 mmol/L  $\text{NH}_4\text{HCO}_3$  solution, denatured with 20 mmol/L dithiothreitol (Sigma) at  $37^{\circ}\text{C}$  for 1 hour, alkylated with 50 mmol/L iodoacetamide (Sigma) in the dark for 30 minutes, washed twice with UA and  $\text{NH}_4\text{HCO}_3$ , and digested with trypsin (Promega) at a 1:50 ratio at  $37^{\circ}\text{C}$  for 14 hours. Peptide mixtures were desalted using HLB columns (Waters) and vacuum-dried.

### High pH Reverse Phase Chromatography Pre-fractionation

Digested samples were resuspended in 0.1% formic acid and diluted to 0.5 g/L. Mixed peptide samples were prepared from each sample and fractionated using a high pH reverse-phase peptide separation kit (Thermo Fisher Scientific). Mixed peptide samples and acetonitrile gradient elution buffers were applied to the column, and ten fractions were collected by centrifugation, vacuum-dried, and resuspended in 0.1% formic acid. iRT synthetic peptides (Biognosis) were added to the ten fractions and each sample at a 10:1 volume ratio.

### LC-MS/MS Data Acquisition

Mass spectrometry analysis was performed using an EASY-nLC 1200 chromatography system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). Peptide samples were loaded onto a trap column ( $75\text{ }\mu\text{m} \times 2\text{ cm}$ , C18, 2  $\mu\text{m}$ , Thermo Fisher) and an analytical reverse-phase column ( $50\text{ }\mu\text{m} \times 15\text{ cm}$ , C18, 2  $\mu\text{m}$ , Thermo Fisher),

eluted at 300 nL/min for 90 minutes with a gradient of 4%-35% mobile phase (80% acetonitrile + 0.1% formic acid + 20% water). Parameters were set as follows: spray voltage 2.4 kV, MS1 full scan range 350-1550 m/z, resolution 60,000; MS2 scan range 200-2000 m/z, resolution 30,000, cycle time 3s, top speed mode, 30% HCD collision energy. The ten fractions from reverse-phase chromatography were analyzed in DDA mode. DDA results were searched against a human database (released in 2018, containing 20,346 sequences) with iRT peptide sequences added using Proteome Discoverer software (version 2.1). Search parameters: trypsin digestion, maximum 2 missed cleavages, precursor mass tolerance 10 ppm, fragment mass tolerance 0.02 Da, methionine oxidation as variable modification, cysteine carbamidomethylation as fixed modification, protein FDR set to 1%. PD search results were used to establish DIA acquisition methods, with window width and number calculated based on m/z distribution density. Individual peptide samples were analyzed in DIA mode with three technical replicates per sample.

### Mass Spectrometry Data Analysis

MS data were processed and analyzed using Spectronaut X software. A spectral library was built from ten DDA raw files, and DIA raw files from each sample were searched against this library. High-confidence proteins were defined by peptide q-value < 0.01, with protein quantification based on the peak area of all fragment ions from secondary peptides.

### Non-invasive Blood Pressure Measurement

At 4 months of age, blood pressure was measured non-invasively using a tail-cuff system. Rats were placed in restrainers and positioned in the fixation frame with tails inserted into pressure cuffs near the tail base, positioning the tail artery directly over the pulse sensor. Measurements were taken after pulse stabilization.

### Brain Tissue Collection and Hematoxylin-Eosin Staining

Rats underwent intracardiac perfusion with 0.9% saline to clear blood, followed by rapid fixation with 4% paraformaldehyde. Whole brains were removed and preserved in 4% paraformaldehyde. HE staining was performed through tissue trimming, dehydration, clearing, paraffin embedding, sectioning, slide mounting, dewaxing, hematoxylin staining, hydrochloric acid-alcohol differentiation, dehydration, eosin counterstaining, and coverslipping.

### Statistical Analysis

Differential proteins were screened by comparing different time points within each rat. Comparisons between two groups were performed using two-tailed unpaired t-tests. Differential proteins were defined by fold change  $FC \geq 1.5$  or  $FC \leq 0.67$ , with  $P < 0.05$ .

## Functional Annotation of Differential Proteins

Differentially expressed proteins were analyzed for functional enrichment using the DAVID database (<https://david.ncifcrf.gov/>) and IPA software (Ingenuity Systems, Mountain View, CA, USA). Statistical significance was set at  $P < 0.05$ .

## Results

### Characteristics of SHRSP Rats

At 4 months of age, tail-cuff blood pressure measurement revealed that SHRSP rats had mean systolic pressure of 140 mmHg and mean diastolic pressure of 180 mmHg, compared to 110 mmHg systolic and 130 mmHg diastolic in control rats. SHRSP rats showed significantly higher systolic ( $P < 0.01$ ) and diastolic ( $P < 0.001$ ) pressures than controls (Figure 2), confirming the hypertensive state. At 14 months, SHRSP rats exhibited behavioral changes including prone immobility, inability to walk, and depressive symptoms. After euthanasia with excess anesthesia, brain and kidney tissues from three rats were subjected to HE staining. Figure 3 shows pathological findings from three 14-month-old SHRSP rats. Rat #1 (Figures 3A-B) showed no brain hemorrhage foci; kidneys displayed minimal glomerular and tubular swelling, degeneration, fibrosis, and occasional protein casts. Rat #2 (Figures 3C-D) exhibited hemorrhagic foci in the hippocampus and periventricular regions with liquefactive necrosis of surrounding brain tissue; kidneys showed multiple necrotic and atrophic glomeruli and tubules, some with hyaline degeneration and fibrosis, thickened renal arteriole walls with luminal narrowing, perivascular inflammatory cell infiltration, and frequent protein casts in tubules. Rat #3 (Figures 3E-F) showed multiple scattered hemorrhagic foci in cortical and hippocampal regions causing sclerotic necrosis of surrounding brain tissue; kidneys displayed multiple necrotic and atrophic glomeruli and tubules, some with hyaline degeneration and fibrosis, thickened renal arteriole walls with luminal narrowing, perivascular inflammatory cell infiltration, and frequent protein casts in tubules. These pathological results demonstrate that 14-month-old SHRSP rats develop both cerebral hemorrhage lesions and kidney pathology.

### Urinary Proteome Changes in SHRSP Rats

We collected urine samples from four rats at six time points (1, 4, 8, 10, 12, and 14 months), extracted urinary proteins, digested them, and performed data-dependent mass spectrometry acquisition with three technical replicates per sample. Protein quantification was performed using Spectronaut software. A total of 884 proteins were identified, with 649 proteins retained for subsequent analysis after missing value imputation. Differential proteins were screened across time points for each rat using criteria of fold change  $\geq 1.5$  or  $\leq 0.67$  and  $P < 0.05$ . Recognizing that pathological results indicated individual variation in disease progression, we employed a within-subject comparison approach to bet-

ter understand disease progression dynamics and identify potential intervention targets.

For Rat #1, comparing across six time points yielded 173 differential proteins at month 4 versus month 1, 321 at month 8 versus month 4, 91 at month 10 versus month 8, 61 at month 12 versus month 10, and 52 at month 14 versus month 12. Venn diagram analysis revealed 2 differential proteins common across all time points (Figure 4A). For Rat #2, we identified 176 differential proteins at month 4 versus month 1, 302 at month 8 versus month 4, 128 at month 10 versus month 8, 113 at month 12 versus month 10, and 65 at month 14 versus month 12, with 3 proteins common across all time points (Figure 4B). For Rat #3, we found 113 differential proteins at month 4 versus month 1, 380 at month 8 versus month 4, 91 at month 10 versus month 8, 69 at month 12 versus month 10, and 41 at month 14 versus month 12, with 4 proteins common across all time points (Figure 4C). For Rat #4, we identified 126 differential proteins at month 4 versus month 1, 214 at month 8 versus month 4, 2611 at month 10 versus month 8, 29 at month 12 versus month 10, and 95 at month 14 versus month 12, with 3 proteins common across all time points (Figure 4D).

### Functional Analysis of Differential Proteins at Different Time Points

Functional annotation analysis was performed using DAVID and IPA software, categorizing differential proteins from each rat by biological process and signaling pathway.

For Rat #1, differential proteins identified at months 4, 8, and 14 were analyzed. In biological processes (Figure 5A), cellular oxidative detoxification, proteolysis, aging, and fibrinolysis were significantly enriched across all three time points. Complement activation (classical pathway), oxidative stress response, negative regulation of endopeptidase activity, and drug response were enriched at months 4 and 8. Response to organic substances and response to nutrient levels were enriched at months 4 and 14. Glycolytic process, acute-phase response, oxidation-reduction process, and phagocytosis were enriched only at month 4. Blood coagulation, cell adhesion, negative regulation of blood coagulation, inflammatory response, and epithelial cell differentiation were enriched only at month 8. Intermediate filament organization, angiotensin regulation, negative regulation of ERK1 and ERK2 cascade, and renin-angiotensin regulation of systemic arterial blood pressure were enriched only at month 14. In signaling pathways (Figure 6A), LPS/IL-1 mediated inhibition of RXR function and iron homeostasis signaling were enriched at months 4 and 8. LXR/RXR activation and coagulation system were enriched at months 8 and 14. Apelin adipocyte signaling pathway was enriched only at month 4. Atherosclerosis signaling was enriched only at month 8. Glucocorticoid receptor signaling was enriched only at month 14.

For Rat #2, differential proteins at months 4, 8, and 14 showed enrichment of proteolysis and retina homeostasis across all three time points in biological

processes (Figure 5B). Acute-phase response, negative regulation of endopeptidase activity, response to organic substances, complement activation (classical pathway), hydrogen peroxide catabolic process, and organ regeneration were enriched at months 4 and 8. Fibrinolysis and blood coagulation were enriched at months 4 and 14. Response to lead ion, drug response, response to nutrient, and oxidation-reduction process were enriched only at month 4. Aging, negative regulation of blood coagulation, heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules, cellular oxidative detoxification, response to nutrient levels, and response to oxidative stress were enriched only at month 8. Intermediate filament organization, defense response to Gram-negative bacterium, receptor-mediated virion attachment to host cell, regulation of tissue remodeling, and negative regulation of cysteine-type endopeptidase activity involved in apoptotic process were enriched only at month 14. In signaling pathways (Figure 6B), proteolysis and retina homeostasis were enriched across all time points. Negative regulation of endopeptidase activity and complement activation classical pathway were enriched at months 4 and 8. Fibrinolysis and coagulation pathways were enriched at months 8 and 12.

For Rat #3, differential proteins at months 4, 8, and 14 showed enrichment of acute-phase response, negative regulation of endopeptidase activity, cellular oxidative detoxification, complement activation (classical pathway), hydrogen peroxide catabolic process, and response to hypoxia at months 4 and 8 in biological processes (Figure 5C). Proteolysis, fibrinolysis, and response to nutrient were enriched at months 4 and 14. Chromatin silencing, innate immune response, response to hydrogen peroxide, phagocytosis, recognition, and cellular response to interleukin-6 were enriched only at month 4. Aging, blood coagulation, carbohydrate metabolic process, glutathione metabolic process, negative regulation of blood coagulation, response to lead ion, cell adhesion, and response to oxidative stress were enriched only at month 8. Intermediate filament organization and receptor-mediated endocytosis were enriched only at month 14. In signaling pathways (Figure 6C), complement activation classical pathway, hypoxia response, and glucocorticoid receptor signaling were enriched at months 4 and 8. Aldosterone signaling in epithelial cells and Apelin adipocyte signaling pathway were enriched at month 14.

For Rat #4, differential proteins at months 4, 8, and 14 showed enrichment of proteolysis and oxidation-reduction process across all three time points in biological processes (Figure 5D). Response to organic substances, drug response, aging, and complement activation (classical pathway) were enriched at months 4 and 8. Negative regulation of endopeptidase activity, fibrinolysis, blood coagulation, acute-phase response, and negative regulation of blood coagulation were enriched at months 8 and 14. Chromatin silencing, cell adhesion, cellular oxidative detoxification, response to oxidative stress, phagocytosis engulfment, cytoskeleton organization, and positive regulation of B cell activation were enriched only at month 4. Glucocorticoid response, response to organic cyclic compounds, inflammatory response, positive regulation of phagocytosis, and response to hypoxia were enriched only at month 8. Carbohydrate metabolic

process, response to nutrient, negative regulation of inflammatory response, and phosphatidylcholine metabolic process were enriched only at month 14. In signaling pathways (Figure 6D), proteolysis and oxidation-reduction process were enriched at month 4. Glucocorticoid response, renin-angiotensin signaling pathway, and Apelin adipocyte signaling pathway were enriched at month 8.

## Discussion

In this study, we employed the SHRSP rat model and label-free quantitative proteomics to identify dynamic changes in the urinary proteome at different time points during stroke-prone hypertension progression. We identified enrichment of important biological processes including renin-angiotensin regulation of systemic arterial blood pressure, response to oxidative stress, blood coagulation, fibrinolysis, intermediate filament organization, complement activation classical pathway, cellular response to interleukin-6, inflammatory response, cytokine response, and regulation of phagocytosis. Beyond elevated blood pressure, renal oxidative stress induced by the renin-angiotensin-aldosterone system and vascular inflammation are established pathophysiological mechanisms in hypertension. First, hypertension is characterized by persistent high pressure in systemic arteries, and urinary angiotensinogen serves as an indicator of intrarenal angiotensin status, with studies showing elevated urinary angiotensinogen levels in hypertensive patients. Increased arterial pressure is transmitted to the kidneys, enhancing sodium and water excretion, with the renin-angiotensin-aldosterone system playing a central role in maintaining pressure-natriuresis. Second, oxidative stress is implicated in hypertension pathogenesis, promoting endothelial dysfunction, vascular remodeling, and inflammation that lead to vascular injury. Clinical studies in essential hypertension demonstrate positive correlations between blood pressure and oxidative stress biomarkers and negative correlations with antioxidant levels. Third, inflammation plays a crucial role in hypertension development and maintenance, involving activation of various immune cells including T cells, antigen-presenting cells, macrophages, natural killer cells, and B cells. Pro-inflammatory cytokines exacerbate cardiac hypertrophy and fibrosis, causing vascular dysfunction and organ damage in hypertension. Hypertension also triggers inflammatory responses and functional impairment in the brain, providing new targets for hypertension regulation. IL-6 is a multifunctional pro-inflammatory cytokine, and studies suggest IL-6 production may contribute to hypertension in Dahl salt-sensitive rats by mediating macrophage infiltration or proliferation in kidneys. Additionally, clinical research has found that IL-6 levels correlate with poor functional outcomes after spontaneous intracerebral hemorrhage, suggesting potential as a therapeutic target.

Furthermore, differential proteins were enriched in several important pathways, including aldosterone signaling in epithelial cells, renin-angiotensin signaling, IL-12 signaling and production in macrophages, Apelin adipocyte signaling pathway, calcium transport pathway, STAT3 pathway, and glucocorticoid receptor signaling. Aldosterone, a steroid hormone synthesized and secreted by the

adrenal cortex zona glomerulosa, regulates sodium homeostasis, blood volume, and blood pressure. Its excessive secretion causes hypertension and increases morbidity and mortality, making understanding aldosterone biosynthesis regulatory pathways valuable for identifying new therapeutic targets for cardiovascular diseases including hypertension. ACE inhibitors target ACE expressed in endothelial cells of large and small vessels, capillaries, venules, and pulmonary endothelium, catalyzing conversion of angiotensin I to angiotensin II, thereby dilating small resistance arteries and reducing total peripheral resistance and blood pressure. ARBs antagonize angiotensin II effects at the angiotensin II receptor type 1 level, reducing angiotensin II-induced vasoconstriction, sodium retention, and aldosterone release, leading to vasodilation of small resistance arteries and decreased blood pressure. During hypertension, monocyte activation by vascular endothelium may result from loss of NO signaling, increased release of IL-6 and hydrogen peroxide from dysfunctional endothelial cells, and STAT activation in adjacent monocytes, suggesting that interventions enhancing bioavailable NO, reducing IL-6 or hydrogen peroxide production, or inhibiting STAT3 may have anti-inflammatory effects in hypertension and related disorders. Calcium channel blockers inhibit extracellular calcium influx through ion-specific channels across cell membranes; currently available CCBs inhibit L-type channels in humans, causing vascular smooth muscle cell relaxation, vasodilation, and blood pressure reduction when inward calcium flow is inhibited. Apelin is a vasoactive peptide, and its receptor APJ is widely expressed in cardiovascular tissues including blood vessels, heart, and brain regions involved in cardiovascular regulation. Accumulating evidence indicates that the apelin/APJ receptor system plays regulatory roles in cardiovascular physiology and pathophysiology, making it a potential target for cardiovascular drug discovery and development. Glucocorticoids participate in blood pressure regulation through various extrarenal tissues including vascular smooth muscle, vascular endothelium, central nervous system, and adipose tissue, with excessive glucocorticoids inducing hypertension through promiscuous activation of mineralocorticoid receptors. Glucocorticoid receptors are widely expressed in organ systems involved in blood pressure regulation and play important roles in hypertension pathogenesis and maintenance. These results suggest that urine protein-associated signaling pathways may provide new targets for hypertension drug research.

The duration of hypertension development and its age-relatedness present challenges requiring long-term longitudinal studies in humans. Considering variable disease progression in animal models, our within-subject comparison approach represents a valuable strategy for studying urinary proteomics in multifactorial complex diseases. We identified several important biological processes and pathways commonly detected across multiple rats, suggesting that significant pathways in hypertension may be limited in number. Unique biological pathways in individual rats may provide new clues for personalized hypertension diagnosis and treatment. In the future, hypertensive patients could undergo urinary proteome analysis for pathway profiling to guide selection of targeted drugs for personalized therapy.

This study demonstrates that urinary proteomics can reflect pathological mechanisms associated with hypertension. This suggests that urinary proteomic approaches may be applied to investigate hypertension mechanisms, identify novel drug targets, and potentially provide personalized antihypertensive treatment strategies with optimal efficacy.

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**Corresponding author:** Youhe Gao, E-mail: gaoyouhe@bnu.edu.cn

**Author Contributions:**

Youhe Gao and Wenshu Meng: Conceived the study and designed the research;

Wenshu Meng: Performed the experiments;

Wenshu Meng: Collected, processed, and analyzed the data;

Youhe Gao and Wenshu Meng: Wrote the manuscript;

Youhe Gao and Wenshu Meng: Revised the final version of the paper.

*Note: Figure translations are in progress. See original paper for figures.*

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