Original Article
Abnormal expression of proteins in human renal mesangial cells stimulated by high levels of uric acid

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Abstract: High levels of uric acid (UA) could lead to serious renal disease. However, the underlying mechanisms are not very clear yet. This study used human renal mesangial cells (HRMC) as a model and found that cell proliferation of HRMC was significantly suppressed with increasing concentrations of UA in culture medium. Furthermore, a mass spectrometry-based proteomics approach was used to measure changes of proteins with and without treatment of UA. Among 1,977 proteins quantified, 314 were abnormally expressed in the stimulation of UA. Bioinformatics revealed that differential proteins were enriched in the pathways of endoplasmic reticulum (ER) stress and apoptosis. Several key proteins were further verified by Western blotting assay. Flow cytometry experiments also confirmed the effects of UA on apoptosis of HRMC. Finally, this study discussed the mechanisms underlying apoptosis pathways promoted by UA in HRMC. This work provides an overview of protein expression changes in HRMC cells treated by high levels of UA and potentially contributes to further study of cell apoptosis in renal disease patients.

Keywords: Human renal mesangial cell, apoptosis, renal disease, proteomics, uric acid

Introduction
Uric acid (UA) has long been considered an inert end product of purine catabolism in mammals [1, 2]. It is not only an important indicator of physiological state but also the cause of some diseases. For example, high levels of UA in blood can cause hyperuricemia, which has emerged as a major health problem in industrialized nations. Epidemiological evidence further demonstrates that prevalence of hyperuricemia is increasing worldwide. It has been estimated that 5-10% of adult Americans suffer from hyperuricemia. In Asia, it is 26.1% for men and 17.0% for women [3]. Hyperuricemia can not only cause renal insufficiency but is also associated with end-stage renal disease [4]. A substantial body of evidence has suggested that chronic hyperuricemia is an independent risk factor for occurrence and development of kidney disease. However, the contribution of UA levels to renal injury and the possible underlying mechanisms are not completely understood. At present, the study of UA damage has focused on smooth muscle cells [5], vascular endothelial cells [6], and urate deposition in renal interstitial damage. However, the effects of UA on glomerular mesangial cells (GMC) remain unclear.

Proteins, as actual executives in all cellular processes, have been thought to be key factors for understanding disease mechanisms [7]. Abnormal expression of proteins is always associated with occurrence and development of disease. Mass spectrometry (MS) has become a powerful tool for proteomics measurement. Comprehensive proteomic analyses, thus, substantially contribute to the understanding of complex protein pathways, providing potentially valuable evidences as biological drivers for disease investigation. This present study performed comparative proteomic analysis of human renal mesangial cells (HRMC) of two different cultures (elevated UA or not) to provide clues on molecular mechanisms underlying biological differences observed between them. The use of a highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach allowed this study to identify thou-
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Thousands of proteins in each HRMC cell sample. Next, 314 abnormally expressed proteins were identified. This study further analyzed their function, pathways, and interaction network. It was found that they enriched in cell apoptosis and ER stress. Flow cytometry experiments were further conducted to characterize the apoptosis phenomenon. Therefore, this study discussed the mechanisms of cell apoptosis induced by high levels of UA.

Materials and methods

Main reagents

Human renal mesangial cell line HRMC (Guangzhou Ginio Biological Technology Co., Ltd.), uric acid (Sigma, USA), monoclonal Ab against CANX, STK3, and HSP B1 were purchased from Abcam (Cambridge, UK). Total CASP3 and cleaved CASP3 were purchased (CST, USA) for use in this study.

Cell culture

Human renal mesangial cells were cultured in Gibco 1640 supplemented with 10% FBS (Gibco, USA), streptomycin (100 g/mL), and penicillin (100 units/mL), at 37°C in 95% air/5% CO₂. Cells were cultured in Gibco 1640 containing either 0.7 mmol/L UA or not for 48 hours.

HRMC protein sample preparation

The cells were plated to 10 cm dishes and either 0.7 mM UA or not was used to stimulate HRMC for 48 hours. Then, cells were harvested respectively. RIPA lysis buffer (containing 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 5 mM sodium butyrate, 50 mM nicotinamide, 50 × Roche Cock tail) was used to extract protein. Protein concentration was determined by BCA method (Thermo scientific, U.S.A). After protein precipitation by TCA, the pellet was resuspended in 100 mM NH₄HCO₃ along with overnight digestion with trypsin (Promega, Madison, WI) with 1:50 (w:w) enzyme to substrate ratio at 37°C. Next, peptides with 5 mM dithiothreitol were incubated at 56°C for 1 hour. After cooling to room temperature, the peptides were alkylated by 15 mM iodoacetamide in a dark room for 45 minutes. The resulting peptides were dried and cleaned with C18 ZipTips (Millipore Corp.), according to manufacturer instructions, prior to nano-HPLC/mass spectrometric analysis.

LC-MS/MS analysis

Analysis was performed in triplicate. Each sample of peptides was reconstituted in 7 µL of HPLC buffer A (0.1% (v/v) formic acid in water) and 5 µL was injected into a Nano-LC system (EASY-nLC 1000, Thermo Fisher Scientific, Waltham, MA). Each sample was separated by a C18 column (50 µm inner-diameter × 15 cm, 2 µm C18) with a 105-minute HPLC-gradient (linear gradient from 2 to 35% HPLC buffer B 0.1% formic acid in acetonitrile in 85 minutes and then to 90% buffer B in 10 minutes). HPLC elute was electrosprayed directly into an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The source was operated at 1.8 kV. Mass spectrometric analysis was carried out in data-dependent mode with an automatic switch between a full MS scan and an MS/MS scan in the orbitrap. For full MS survey scan, automatic gain control (AGC) target was 3e6, scan range was from 400 to 1350, with a resolution of 70,000. The 10 most intense peaks with charge state 2 and above were selected for fragmentation by higher-energy collision dissociation (HCD) with normalized collision energy of 27%. MS2 spectra were acquired with 17,500 resolution. Exclusion duration for data-dependent scans was 30 sec, while the repeat count was 2.

Database search and bioinformatics analysis

Data analysis: Resulting MS/MS data were searched against UniProt database using PD software (v1.5.2.8) with an overall false discovery rate (FDR) for peptides of less than 1%. Peptide sequences were searched using trypsin specificity, allowing a maximum of two missed cleavages.

Bioinformatics analysis: Categorical annotation was supplied in the form of Gene Ontology (GO) biological process (BP) and cellular component (CC). Distribution of different proteins in metabolic pathways was by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Protein-protein interactions were performed by STRING.
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Figure 1. UA decreases cell viability in human renal mesangial cells. A. HRMCs were treated with 0.1 mM, 0.3 mM, 0.5 mM, 0.7 mM UA, or not are shown in column diagram, respectively. The growth rate of HRMCs treated with UA was lower than control. B. HRMCs treated with or without 0.7 mM UA are shown in red and blue, respectively. The growth rate of HRMCs treated with UA was lower than control. Data shown as mean ± SEM; *p < 0.05 vs. normal control.

Figure 2. Identification of differential expression proteins by mass spectrometry and their pathway analysis. A. A total of 1,977 proteins were identified, of which 314 proteins were differentially expressed. Of all differently expressed proteins, 186 proteins were upregulated, while 128 proteins were downregulated. B. The distribution of different proteins in cellular components.

Cell proliferation and apoptosis assay

Cells were planted into 96-well plate at a concentration of 3,000 cells per well and stimulat-ed by 0.1 mM UA, 0.3 mM UA, 0.5 mM UA and 0.7 mM UA, or not. Cell proliferation activity was determined at 6, 12, 24, 48 and 72 hours after stimulation by CCK-8 kit, according to protocol provided by the manufacturer.

To quantify apoptotic cells, flow cytometry experiments were conducted, according to manufacturer instructions. Data were processed with Cellquest software. The numbers present in the four quadrants represent percentages of viable (lower left), necrotic (upper left), early apoptotic (lower right), and late apoptotic (upper right).

Western blot analysis

RIPA lysis buffer (containing 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 5 mM sodium butyrate, 50 mM nicotinamide, 50 × Roche Cock tail) was used to extract protein. The lysate of cells was taken for determination of the concentration of protein with a BCA kit. About 30 μg protein were loaded for 12% SDS-PAGE electrophoresis. After electrophoresis for 1.5 hours, proteins in the SDS-PAGE gel were transferred to nitrocellulose membranes at 200 mA for 1 hour. Membranes were blocked in 5% BSA or 5% milk for 2 hours. Next, the membranes were incubated with a primary antibody against β-actin (1:1000, ZSGB-BIO, China), total CASP3 (1:10000), cleaved CASP3 (1:10000), STK3 (1:10000), HSP B1 (1:1000), or CANX (1:1000) at 4°C overnight, respectively. Afterward, they were incubated with 1:10000 rabbit IgG (ZSB-G-BIO, China) as secondary anti-
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Table 1. Differential proteins related to cell apoptosis

<table>
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<tr>
<th>Accession</th>
<th>Ratio-A/B</th>
<th>Gene name</th>
<th>Description</th>
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<td>Isoform 2 of DNA mismatch repair protein Msh2</td>
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<td>Serine/threonine-protein kinase PAK</td>
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<td>HSPB1</td>
<td>Heat shock protein beta-1</td>
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<td>CUL2</td>
<td>Cullin-2</td>
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<td>[ACTN4</td>
<td>Alpha-actinin-4</td>
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<td>1433E</td>
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<td>STK3</td>
<td>Serine/threonine-protein kinase 3</td>
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Body for 1.5 hours. Blots were developed with BCL reagent (Merck Millipore) and exposed to X-ray film.

Statistical analysis

All data are expressed as mean ± S.D. Statistical analyses were performed using 1-way ANOVA plus Student-Newman-Keuls (SNK-q) test with p < 0.05 considered statistically significant.

Results

Cell proliferation assay

To investigate the effects of UA, HRMCs were treated by different concentrations of UA. As shown in Figure 1A, proliferation of HRMCs was suppressed with increasing concentrations of UA. It was observed that it was changed but no excessive toxicity was induced at 0.7 mmol/L UA. Thus, high levels of UA were used for the following experiment. Next, HRMCs status at different times was observed. As shown in Figure 1B, HRMCs proliferation was significantly suppressed in elevated UA at 48 hours, with no obvious changes in untreated cells. This study, therefore, focused on HRMC at that stage.

Identification and quantification of HRMCs proteins

A total of 1,977 proteins were quantified. Of these, 314 differential proteins were identified. Proteins with ratios ≥ 2 were thought to be upregulated while proteins with ratios < 0.5 were thought to be downregulated. There were
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186 upregulated proteins and 128 downregulated proteins, as shown in Figure 2A. GO and KEGG pathway analysis showed that differential proteins were mainly localized within the nucleus, mitochondria, endoplasm reticulum (ER), and cytoplasm (Figure 2B), and the protein-protein networks showed that many proteins interacted with each other (including some apoptotic proteins) (Supplementary Figure 1).

**Cell apoptosis assay**

HRMC was cultured in normal culture medium and 0.7 mM UA culture medium. After 48 hours, it was found that there were 29 differential proteins related with apoptosis processes (Table 1). Of these, 21 proteins were upregulated, including STK3 (Serine/threonine-protein kinase 3), BAG6 (Large proline-rich protein BAG6), and CANX (Calnexin) which were upregulated 18.24 fold, 2.42 fold and 2.56 fold, respectively. Additionally, 8 proteins were downregulated, including HSP B1 (Heat shock protein beta-1) which was downregulated 5 fold. Expression levels of STK3, CANX, and cleaved caspase-3 tested by Western blot in the 0.7 mM UA group were obviously higher than that in the control group (Table 1). Expression levels of HSPB1 and total caspase-3 tested by Western blot in the 0.7 mM UA group were obviously lower than that in the control group (Figure 3A, 3B). Early apoptosis and late apoptosis rates of 0.7 mM UA group were 9.62% and 9.47% respectively, while the early apoptosis and late apoptosis rates of the control group were 1.5% and 1.63%, respectively (Figure 3C).

**Discussion**

Mass spectrometry (MS)-based proteomics has developed rapidly in recent years and its quantitative accuracy has improved dramatically. This present study carried out a proteomics approach to explore differentially expressed protein profiles of HRMC stimulated by elevated UA or not. It was found that many differential proteins were associated with apoptosis. Next, Western blotting assay was carried out for key proteins in the apoptosis processes, including STK3, HSP B1, and CANX. STK3, a pro-apoptotic cytoplasmic kinase belonging to the Ste-20 kinase family, is one of the core elements of mammalian Hippo pathway that controls cell apoptosis and various stress responses [8]. HSP B1, a molecular chaperone belonging to the small shock protein group, has also been identified as an anti-apoptotic factor which confers a survival advantage to cells by reduction of apoptosis [9, 10]. Results indicate that high levels of UA can induce HRMCs apoptosis by both downregulated anti-apoptotic proteins and upregulated pro-apoptotic proteins. Flow cytometry experiments further confirmed that UA may initiate occurrence of HRMC apoptosis.

Next, this study analyzed differential proteins for functional classification, protein-protein ne-
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![Diagram](image)

**Figure 4.** Pathways involved in the apoptosis of HRMC cells induced by elevated UA.

In conclusion, in this study, cell proliferation of HRMC was significantly suppressed with increasing concentrations of UA in a culture medium. Furthermore, LC-MS/MS analysis was used to profile differentially expressed proteins in HRMCs treated with elevated UA. Results showed that abnormally expressed proteins enriched in the pathways of endoplasmic reticulum (ER) stress and apoptosis. This work suggests that high levels of uric acid induce HRMC apoptosis through ER stress induction and caspase pathways. These findings may offer potential clues regarding underlying mechanisms of hyperuricemia toxicity in the kidneys.

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**Disclosure of conflict of interest**

None.

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Supplementary Figure 1. The interaction network of differential proteins associated with apoptosis in HRMC treated by UA.