Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;27:91-98

Accepted: January 19, 2011

Differentially Expressed Protein Profile of Renal Tubule Cell Stimulated by Elevated Uric Acid Using SILAC Coupled to LC-MS

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Key Words
SILAC • Uric Acid • Renal tubule epithelial cell

Abstract
Background/Aims: Hyperuricemia could lead to serious renal disease. It will advance our understanding of the mechanism of this disease to study the differentially expressed protein in renal tubular epithelial cells stimulated by elevated uric acid. Methods: We used SILAC coupled to LC-MS to study differentially expressed protein profile and to analysis the functional status of renal tubular epithelial cells stimulated by 600μM uric acid, and to investigate the potential biology function. The MS results were analyzed by online platform and further confirmed by western blotting. Results: 789 differentially expressed proteins were identified, of which 42 proteins and 49 proteins were related with cell proliferation and cell apoptosis, respectively. Pathway analysis showed that MAPK signaling pathway was a key pathway related to the function of these proteins. In addition, prohibitin-2 was identified to be related to renal cell transdifferentiation stimulated by elevated uric acid. Conclusion: This work provides an overview of protein expression changes in HK-2 cells treated with elevated uric acid and will contribute to further study of cell apoptosis and fibrosis in renal disease patients.

Introduction

Hyperuricemia is a common disease, which can cause direct damage to kidney and cardiovascular system [1, 2]. Hyperuricemia can not only cause renal insufficiency [3], but also is associated with end-stage renal disease [4]. Human serum uric acid mainly exists as free acid in blood and can freely filter through glomerulus. In hyperuricemia patients, uric acid could be transported into renal tubule by a complex mechanism and lead to the renal tubule epithelial atrophy [5]. Elevated uric acid will suppress the progression of renal tubular epithelial cells. It was reported that cell apoptosis was clinically observed in renal tubule of familial uric acid nephropathy patients [6, 7]. However, it remains elusive that how many differentially expressed genes and proteins involved in cell progression and cell apoptosis in renal tubule when uric acid level was elevated. Therefore it is necessary to study differentially expressed genes by high-throughput method under the condition of

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elevated uric acid.

Previous MS analysis is restricted in qualitative analysis about differentially expressed proteins, while SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture) analysis firstly reported by Mann group can be used to both qualitatively and quantitatively analyze differentially expressed proteins and greatly improves the sensitivity and accuracy of measurement [8]. In addition, this new technique has some other advantages as listed: protein saving, high efficiency labeling, error inefficiency, multiple sample comparison, high percentage of peptide coverage, and simple operation [9].

Here we used HK-2 cell line to high-throughput and effectively screen the differentially expressed proteins of renal tubule under elevated uric acid by SILAC coupled to LC-MS analysis. We also analyzed proteins involved in cell proliferation and cell apoptosis using http://www.uniprot.com and http://bioinfo.capitalbio.com/mas/ online analysis platforms.

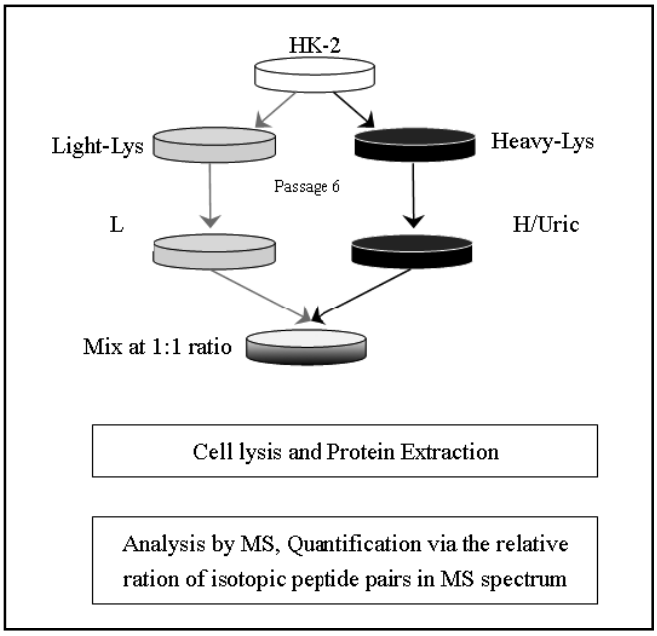

Fig. 1. Schematic overview of SILAC based differentially expressed protein profile in HK-2 cells treated with elevated uric acid or not.

Materials and Methods

Main reagents

Human renal tubular epithelial cell line HK-2 CRL22190 (ATCC, USA), SILAC ™ Kits (Invitrogen, USA), Trypsin and Uric Acid (Promega, USA), Antibodies (SantaCruz, USA), Nano-ESI LTQ-ion trap Mass spectrometer (Thermo Fisher Scientific, Waltham, USA).

Cell culture

Human renal tubular epithelial cell line HK-2 was maintained in a petri dish in DMEM medium with 10% fetal bovine serum. The cultures were subcultured when growing to 80% at 37° C in a 5% CO_2 atmosphere.

Stable heavy isotope labeling

DMEM medium containing stable heavy isotope labeled [$^{13}C_6$]-Lys or light Lys was prepared according to SILAC kit mannual (http://toolszh.invitrogen.com/content/sfs/manuals/silac_man.pdf). HK-2 was serially subcultured for 6 passages to obtain greater than 99% incorporation of the isotope labeled Lys into proteins. The cells are planted to 10 cm dishes and named light DMEM group and heavy DMEM stimulated by uric acid group, respectively. Based on the foundation of our previous studies, 500μM uric acid was used to stimulate renal tubular epithelial cells for 48 hours and the cells were subsequently collected and counted. Cells from the two different groups were mixed together by 1:1 ratio. The general experimental procedure was shown in Fig. 1.

Protein preparation

The protein concentration was determined by BCA method (Bio-Rad Company, U.S.A). 100μg protein with 10 mM DTT was incubated at 56°C for 1 hour. After cooling to room temperature, protein was alkalyted by 50 mM IAM in dark room for 45 minutes. Excess IAM was removed by 40 mM DTT at room temperature. The sample was further dilute to 5 folds by 25mM NH_4HCO_3 and one percent trypsin was sequentially added to digest the protein for 12 hours and 6 hours at 37°C respectively. 0.1% FA was used to end the digestion and the debris was removed by centrifugation for 10 minutes at 13 000 g. The supernatant was stored at -80°C for MS analysis.

LC-MS analysis

LC-MS was performed as reported by JohnYates using LTQ ion-Trap MS (ThermoFisher Scientific, Waltham, USA) [10-12]. 100μg digested peptides were injected onto a self-made biphasic capillary column by high pressure nitrogen facility. The mobile phase consists of A (5% CAN, 0.1% FA), B (80% CAN, 0.1% FA), and C (800 mM ammonium acetate, 5% CAN, 0.1% FA). The biphasic capillary column was disalted by A for 45 minutes. Then the column was washed by C in a step by step manner with the concentration of ammonium acetate from 0 to 800 mM. All the elution from the column was directly loaded onto C18 analytical capillary chromatography column, and fraction from the column was ionized by electrospray for MS analysis. The spray voltage was set to 2.0 kV, and the temperature of the heated capillary was set to 200°C. MS data was collected in a data dependent acquisition mode. Parameters related with MS/MS data acquisition were set as follows: normalized collision energy 35%, ion selection threshold 200 counts, activation Q 0.250, activation time 30ms, and dynamic exclusion time 30 seconds. The elution gradient of HPLC and MS scanning were controlled by XCalibur (Thermo Fisher, Waltham, USA).

Data analysis

Data from MS analysis were searched against NCBI RAT Refseq Database,Version 2009.10.05 using SEQUESTv.28 of Bioworks3.31. The false discovery rate was calculated in reverse database using trypsin as searching parameter including up to two missed cleavages [13]. The initial mass deviations of precursor and fragment ions were up to 2 Da and 1 Da, respectively. The mass deviations of amino acids modification was set to 57.02 Da for alkylated cysteine and 19.02Da for oxidated methionine, respectively. The peptide possibility was calculated by Bioworks. The false discovery rate will reach 1% by XCorr, sp, Rsp, DeltaCn and Peptide possibility filtration. The protein coverage rate was calculated by protein Coverage Summarizer.

Protein functional groupings

The Refseq of proteins were transformed into UniProtKB on http://www.uniprot.org website [14]. Pathway networks and protein functional grouping were generated by http://bioinfo.capitalbio.com/mas/ online platform.

Proximal Tubule Epithelial Cell Culture

The primary proximal tubule epithelial cells (PTECs) were prepared and grown in media from proximal tubule fragments isolated from male Wistar rats (weighing 200-250 g), as previously described [15, 16] . Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). After a midline incision had been made, the kidneys were selectively perfused with Krebs-Henseleit buffer with collagenase at a flow rate of 6 ml/min. The kidneys were excised, then the cortexes were removed, minced and digested in buffer with collagen for 2–3 min. The digested suspension was filtered through an 80-μm mesh and subjected to centrifugation on Ficoll density gradient. The proximal tubular suspension was plated in 100-mm diameter dishes and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. The first medium was changed after 72 h of seeding and then every alternate day.

Cell proliferation assay

The cells were planted into 96-well plate at a concentration of 3000 cells per well and stimulated by 600μM uric acid. The cell proliferation activity was determined at 24h, 48h, and 72h after stimulation by CCK-8 kit according to the protocol provided by manufacturer (http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Datasheet/6/96992dat.Par.0001.File.tmp/96992dat.pdf).

Assay Apoptosis by FACS

To quantify the apoptotic cells, flow cytometry experiments were conducted according to our previous study and the manufacturer's instruction. The data were processed with Cellquest software. This test discriminates between intact cells (annexin V–/PI–), apoptotic cells (annexin V+/PI–) and necrotic cells (annexin V+/PI+).

Western Blot assay

RIPA lysis buffer (containing 50 mmol/L Tris-HCl pH 7.5,150 mmol/L NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, a variety of protease inhibitionagents: 1 μg / ml) was used to extract protein. The lysate of cell was taken for determination of the concentration of protein with BCA kit. About 80μg protein were loaded for 10% SDS-PAGE electrophoresis , then transferred to PVDF membrane, it will be kept overnight in the 5% no-milk at 4°C after Ponceau S being dyed. The membrane was incubated in primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were developed with ECL reagent (Santa Cruz Biotechnologies) according to the manufacturer′s instructions and exposed to X-ray film. The protein bands were quantified using Quantity One software (Bio-rad).

Statistical Analysis

The results were presented as means ± SD. The data were analyzed with a one-way analysis of variance plus Bonferroni's correction (Student-Newman-Keuls). A p value less than 0.05 was considered statistically significant.

Results and Discussion

LC-MS analysis results

In total, 22250 hits were identified. About 1329 hits belong to reverse transcription database, while 21921 hits belong to real database. The false discovery rate was 1.47%, which is defined as the ratio between hits from reverse transcription database and real database. 13652 unique peptides (Suppl. 1 peptide.xls, (http://www.rayfile.com/zh-cn/files/242cf64f-24a3-11e0-a3d8-0015c55db73d/)) were identified from 21921 effective peaks. A total of 1745 proteins were generated from 13652 peptides by protein coverage summarizer, of which differently expressed proteins were 789. The proteins with 20>Nratio>1.5 were thought to be up regulated, while proteins with 0.05<Nratio<0.66 were thought to be down regulated. Of all the differently expressed proteins, 92 proteins are absent in Uniprot database, and 697 proteins have 936 hits in Uniprot database. The basic information about proteins and the functional grouping were shown in Suppl. 1 Protein annotation.xls (http://www.rayfile.com/zh-cn/files/242cf64f-24a3-11e0-a3d8-0015c55db73d/).

Functional grouping of differentially expressed proteins

It is reported that elevated uric acid may affect cell proliferation and cell apoptosis [6, 7, 17-19]. Therefore we focus on proteins involved in cell proliferation and cell apoptosis using GO classification system to analyze differentially expressed proteins (Table 1 & Table 2).

It was found that about 42 proteins are related with cell proliferation, of which 12 proteins such as K22E, TRXR1, HDAC1, Prohibitin are downregulated. K22E

Refseq	NRatio (H/L)	Protein AC	Protein ID	Protein Name
NP_000414.2	0.2927481	P35908	*K22E*	Keratin, type II cytoskeletal 2 epidermal
NP_000627.2	2.2414049	P04179	*SODM*	Superoxide dismutase [Mn], mitochondrial precursor
NP_000691.1	2.4099465	P04083	*ANXA1*	Annexin A1
NP_000875.2	1.7134376	P12268	*IMDH2*	Inosine-5'-monophosphate dehydrogenase 2
NP_001001.2	3.5065335	P62753	*RS6*	40S ribosomal protein S6
NP_001010942.1	1.8217269	P61224	*RAP1B*	Ras-related protein Rap-1b precursor
NP_001014796.1	3.5139179	Q16832	*DDR2*	Discoidin domain-containing receptor 2 precursor
NP_001028886.1	3.7926811	P46087	*NOP2*	Putative ribosomal RNA methyltransferase NOP2
NP_001243.1	2.6811872	P32970	*CD70*	CD70 antigen
NP_001311.3	1.5846409	P67870	*CSK2B*	Casein kinase II subunit beta
NP_001354.1	3.3473718	O60832	*DKC1*	H/ACA ribonucleoprotein complex subunit 4
NP_001656.2	0.2015809	P84095	*RHOG*	Rho-related GTP-binding protein RhoG precursor
NP_002120.1	2.1852869	P26583	*HMGB2*	High mobility group protein B2
NP_002511.1	1.6513654	P06748	*NPM*	Nucleophosmin
NP_002736.3	2.6153912	Q1HBJ4	*Q1HBJ4*	Mitogen-activated protein kinase 1
NP_002746.1	1.7427717	Q02750	*MP2K1*	Dual specificity mitogen-activated protein kinase kinase 1
NP_003079.1	3.5612088	Q16658	*FSCN1*	Fascin
NP_003321.3	0.526368	Q16881	*TRXR1*	Thioredoxin reductase 1, cytoplasmic
NP_004604.2	0.5286739	P21980	*TGM2*	Protein-glutamine gamma-glutamyltransferase 2
NP_004740.2	1.9239457	Q969Z0	*TBRG4*	Protein TBRG4
NP_004955.2	0.597647	Q13547	*HDAC1*	Histone deacetylase 1
NP_005337.2	0.0522777	P08107	*HSP71*	Heat shock 70 kDa protein 1A/1B
NP_005403.2	1.9296968	Q5BJF5	*Q5BJF5*	Serine hydroxymethyltransferase
NP_005548.2	0.1886353	P08779	*K1C16*	Keratin, type I cytoskeletal 16
NP_005871.1	2.1013599	O60884	*DNJA2*	DnaJ homolog subfamily A member 2
NP_006182.2	1.6509476	Q9UQ80	*PA2G4*	Proliferation-associated protein 2G4
NP_006212.1	2.1383702	Q13526	*PIN1*	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
NP_006283.1	0.5222558	Q99816	*TS101*	Tumor susceptibility gene 101 protein
NP_006550.1	1.757987	Q07666	*KHDR1*	KH domain-containing, RNA-binding, signal transduction-associated protein 1
NP_009148.2	3.3658769	Q9BUL8	*PDC10*	Programmed cell death protein 10
NP_009204.1	0.2217249	Q99623	*PHB2*	Prohibitin-2
NP_036473.2	2.7436187	Q9BZE4	*NOG1*	Nucleolar GTP-binding protein 1
NP_055118.1	0.5818834	B2RDF2	*B2RDF2*	Pescadillo homolog 1
NP_055317.1	2.4577609	Q9UMS4	*PRP19*	Pre-mRNA-processing factor 19
NP_055439.1	3.6230269	P06703	*S10A6*	Protein S100-A6
NP_057157.1	2.4857092	Q9Y3E1	*HDGR3*	Hepatoma-derived growth factor-related protein 3
NP_066964.1	1.6009956	P13010	*XRCC5*	X-ray repair cross-complementing protein 5
NP_113671.3	0.6538478	Q96J02	*ITCH*	E3 ubiquitin-protein ligase Itchy homolog
NP_874365.2	1.9280579	Q14160	*SCRIB*	Protein scribble homolog
NP_955392.1	1.8372523	O43399	*TPD54*	Tumor protein D54
NP_996562.1	0.2015809	Q9BVP2	*GNL3*	Guanine nucleotide-binding protein-like 3
NP_998811.1	2.2000539	P23381	*SYWC*	Tryptophanyl-tRNA synthetase, cytoplasmic

Table 1. Protein profiles related to cell proliferation in GO Summary

belongs to intermediate filament protein family. As a marker of epithelial cells, K22E plays an important role in epithelial cell proliferation [20]. Overexpression of K22E can enhance the cell adhesion and cell growth of MDCK [21]. TRXR1 can inhibit the production of ROS and play an important role in the oxido-reduction signaling pathway [22]. In this way, TRXR1 could regulate cell proliferation, cell apoptosis, and cell migration. Guo et al. reported that overexpression of Prohibitin can repress cell phenotype change caused by TGF-b by inhibiting the transcription of E2F [23]. Renal pathological variation is mainly interstitial fibrosis in hyperuricemia patients. Then we prepared the primary proximal tubule epithelial cells (PTECs) to confirm Prohibitin-2 protein expression level when stimulated by elevated uric acid. It was found that the expression of Prohibitin-2 was downregulated in PTECs stimulated by elevated uric acid for 48h, which may promote trans-differentiation of renal tubular epithelial cells. MS results showed that the expression of α-SMA, a marker of fibrosis cell, was up-regulated for 3.9 folds, while the expression of E-cadherin, a marker of renal tubule cell, was down-regulated to 17 percent. Western blotting results also showed the up-regulation of α-SMA expression and down-regulation of E-cadherin expression in PTECs when stimulated by elevated uric acid for 48 hours (Fig. 2).

S100A6 is one of the up regulated proteins related with cell proliferation. Kroliczak W et al reported that P53 can suppress cell proliferation by inhibiting the expression of S100A6 [24]. Zimmermann S et al. found

Refseq	NRatio (H/L)	Protein AC	Protein ID	Protein Name
NP_000091.1	3.4433243	Q76LA1	*Q76LA1*	Cystatin-B
NP_000627.2	2.2414049	P04179	*SODM*	Superoxide dismutase [Mn], mitochondrial precursor
NP_000691.1	2.4099465	P04083	*ANXA1*	Annexin A1
NP_000996.2	5.7567393	P23396	*RS3*	40S ribosomal protein S3
NP_000997.1	1.6342083	P61247	*RS3A*	40S ribosomal protein S3a
NP_001093.1	1.6162186	P12814	*ACTN1*	Alpha-actinin-1
NP_001093162.1	0.2895951	Q6IS14	*IF5AL*	Eukaryotic translation initiation factor 5A-1-like
NP_001145.1	2.0657404	P08758	*ANXA5*	Annexin A5
NP_001187.1	0.0957096	A8ASI8	*A8ASI8*	BH3 interacting domain death agonist, isoform CRA_d
NP_001243.1	2.6811872	P32970	*CD70*	CD70 antigen
NP_001531.1	0.4904212	P04792	*HSPB1*	Heat shock protein beta-1
NP_002568.2	2.513613	Q13177	*PAK2*	Serine/threonine-protein kinase PAK 2
NP_002945.1	1.6037776	P62988	*UBIQ*	Ubiquitin
NP_003071.2	0.3007273	O60906	*NSMA*	Sphingomyelin phosphodiesterase 2
NP_003290.1	1.7796684	P14625	*ENPL*	Endoplasmin precursor
NP_003397.1	1.7740179	P63104	*1433Z*	14-3-3 protein zeta/delta
NP_004604.2	0.5286739	P21980	*TGM2*	Protein-glutamine gamma-glutamyltransferase 2
NP_004623.1	3.3802698	P51398	*RT29*	28S ribosomal protein S29, mitochondrial precursor
NP_004740.2	1.9239457	D3DVL6	*D3DVL6*	Transforming growth factor beta regulator 4, isoform CRA_a
NP_004740.2	1.9239457	Q969Z0	*TBRG4*	Protein TBRG4
NP_004936.2	0.5234942	P50570	*DYN2*	Dynamin-2
NP_004955.2	0.597647	Q13547	*HDAC1*	Histone deacetylase 1
NP_004976.2	2.4591257	P01116	*RASK*	GTPase KRas precursor
NP_004997.4	1.8792984	P28331	*NDUS1*	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor
NP_005156.1	0.5551538	P09972	*ALDOC*	Fructose-bisphosphate aldolase C
NP_005304.3	1.6137848	P30101	*PDIA3*	Protein disulfide-isomerase A3 precursor
NP_005337.2	0.0522777	P08107	*HSP71*	Heat shock 70 kDa protein 1A/1B
NP_005720.1	1.9646276	P30405	*PPIF*	Peptidyl-prolyl cis-trans isomerase F, mitochondrial precursor
NP_006098.2	0.381297	O95232	*LC7L3*	Luc7-like protein 3
NP_006294.2	5.3993847	Q13155	*AIMP2*	Aminoacyl tRNA synthase complex -interacting multifunctional protein 2
NP_006729.4	1.9450944	Q12802	*AKP13*	A-kinase anchor protein 13
NP_006776.1	0.1453092	Q9UDY8	*MALT1*	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
NP_009049.2	0.477021	O75962	*TRIO*	Triple functional domain protein
NP_009148.2	3.3658769	Q9BUL8	*PDC10*	Programmed cell death protein 10
NP_036270.1	2.5002482	Q9NY61	*AATF*	Protein AATF
NP_037506.2	1.8861323	Q8WUM4	*PDC6I*	Programmed cell death 6-interacting protein
NP_055535.2	0.5824216	Q9H2G2	*SLK*	STE20-like serine/threonine-protein kinase
NP_055792.1	0.5140313	Q9UKV3	*ACINU*	Apoptotic chromatin condensation inducer in the nucleus
NP_056154.1	0.5429752	Q9H2P0	*ADNP*	Activity-dependent neuroprotector homeobox protein
NP_056656.2	1.8854668	Q59EC0	*Q59EC0*	Adenosine deaminase, RNA-specific isoform ADAR-a variant
NP_061332.2	0.0947523	Q9UHQ4	*BAP29*	B-cell receptor-associated protein 29
NP_066997.3	1.5297571	Q8N163	*K1967*	Protein KIAA1967
NP_071915.1	2.3851052	Q9HA38	*ZMAT3*	Zinc finger matrin-type protein 3
NP_115571.1	4.0711278	P18583	*SON*	Protein SON
NP_115866.1	2.4899676	Q8IXM3	*RM41*	39S ribosomal protein L41, mitochondrial precursor
NP_620116.1	2.2946357	Q07812	*BAX*	Apoptosis regulator BAX
NP_665811.1	1.6819104	O95831	*AIFM1*	Apoptosis-inducing factor 1, mitochondrial precursor
NP_874365.2	1.9280579	Q14160	*SCRIB*	Protein scribble homolog
NP_954657.1	2.0013863	P05783	*K1C18*	Keratin, type I cytoskeletal 18

Table 2. Protein profiles related to cell apoptosis in GO Summary

that the expression of S100A6 was up-regulated in cells lack of telomerase activity [25]. S100A6 may affect the length of telomere by modulating telomerase activity, and overexpression of S100A6 may suppress cell proliferation and even lead to cell senescence. However, Slomnicki LP et al. found that S100A6 could maintain the cell proliferation and function in fibroblast cells [26]. Cell proliferation was suppressed in leukocyte when the expression of S100A6 was knocked down by siRNA [27]. In different types of cells, the effects of S100A6 for cell proliferation differ from each other. Elevated uric acid may suppress cell proliferation by affecting many proteins related with cell proliferation, which is confirmed by the CCK-8 experiment. PTECs proliferation was significantly suppressed after culturing in elevated uric acid for 48 hours (Fig. 3A, $P<0.05$). MS data showed that the expression of HDAC1 was down regulated after elevated uric acid stimulation. HDAC1 belongs to histone deacetylase family and plays an important role in multiple cellular processes. Cell cycle was arrested when the HDAC1 was conditionally knocked out [28, 29]. The down-regulation of this protein gave a reasonable explanation for the phenomenon that cells in G0/G1 phase increase under elevated uric acid condition. No proteins

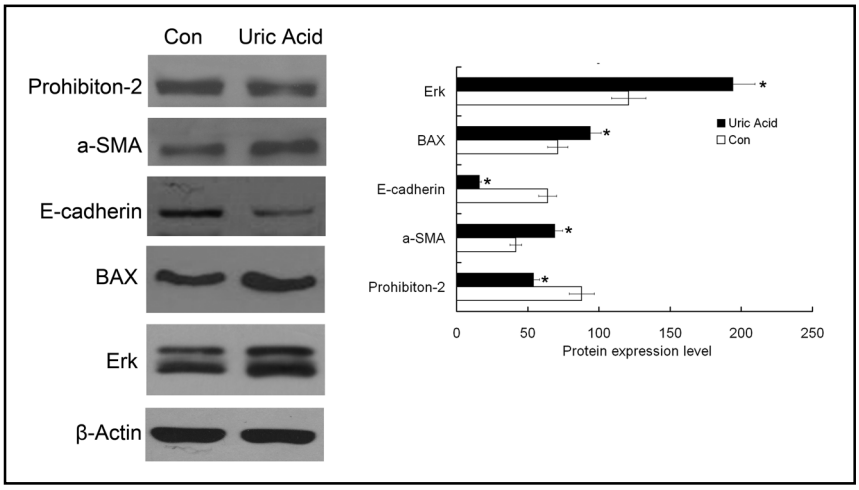

Fig. 2. Western blot analysis of protein expression in PTECs. Cells were treated with elevated uric acid for 48h, then were analysis with Western blot. a-SMA, BAX and Erk were upregulated, while Prohibition-2 and E-cadherin was downregulated in PTECs treated with elevated uric acid, there were significantly difference from those of control. *$p < 0.05$, v.s. control.

related with cell cycle were identified in this study. A possible explanation for this is that our technique is not sensitive enough to test the slight variation of these proteins leading to the change of cell proliferation.

In differentially expressed proteins, 47 proteins are involved in cell apoptosis including BAX and DAP3 that are upregulated by elevated uric acid [30-33]. The expression level of pro-apototic proteins was increased for 3 folds, indicating that elevated uric acid may induce cell apoptosis in renal tubule. The ratio of apoptotic cells is about 10.1±1.1% in cells stimulated by elevated uric acid, which is significantly higher than that in the untreated cells ($P<0.05$)(Fig. 3B). Western blotting results also showed that the BAX expression was up regulated in PTECs after stimulation by elevated uric acid for 48 hours (Fig. 2), which was consistent with the MS results. During ischemic reperfusion injury to the kidney, HSP70 can suppress renal tubule cell apoptosis [34-37]. In addition, Mao H et al found that HSP70 can also prevent renal tubule cell apoptosis and cell transformation induced by TGF-β, indicating that HSP70 may be beneficial for the treatment of obstructive nephropathy by inhibiting renal tubule cell apoptosis and renal pulmonary fibrosis [38]. MS analysis showed that the expression of HSP70 in HK-2 cells treated by uric acid was decreased to 5% of that in untreated cells, while the expression of TGF-β was increased for 2 folds in HK-2 cells treated by uric acid than that in untreated cells. These results indicate that elevated uric acid can induce renal tubule cell apoptosis by both downregulating anti-apoptotic proteins and upregulating pro-apoptotic proteins.

In cells treated with elevated uric acid, the expression of Annexin family was also upregulated, among which Annexin A1 and Annexin A5 are related with cell apoptosis [39-41]. These proteins may induce cell apoptosis and suppress cell proliferation by inhibiting

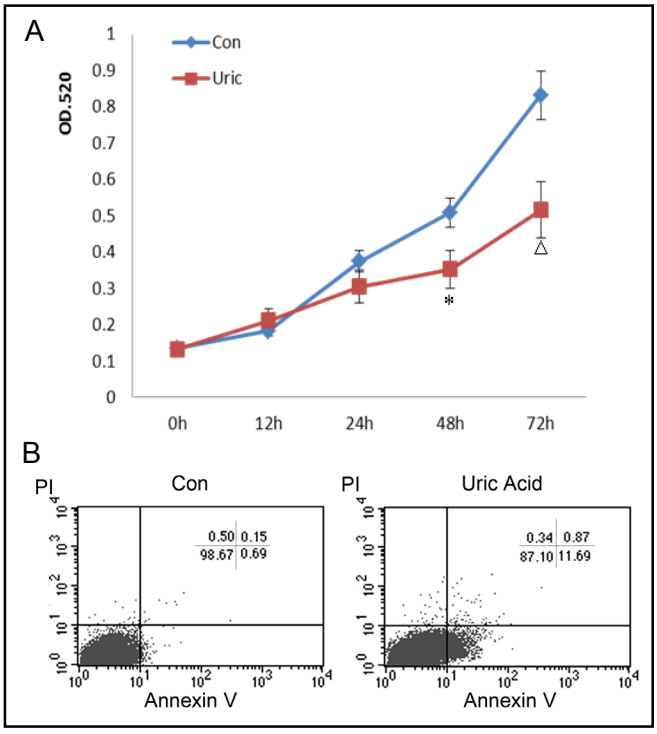

Fig. 3. Suppression of PTEC proliferation and promotion of apoptosis by elevated uric acid. A. PTECs treated with or without 600μM uric acid were shown in red and blue, respectively. The growth rate of PTECs treated with uric acid is lower than control ,*, $\Delta p < 0.05$. B. PTECs treated with or without 600μM uric acid, the percentage of apoptotic cells treated with uric acid were remarkedly higher than control.

NF-κB bond with DNA.

Pathway analysis

Pathway networks, in which the differentially expressed proteins are involved, were generated using MAS system. As depicted in Suppl. 2 (http://

www.rayfile.com/zh-cn/files/ce19c991-24a1-11e0-a9d6-0015c55db73d/), MAPK pathway is located in the center of the networks, which plays an important role in renal tubule cell proliferation and apoptosis. We identified 19 differentially expressed proteins involved in this signaling pathway such as RAC1, MAP4K1 (Table 3), and the western blot results also confirmed that when PTECs were stimulated with 600ìM uric acid for 48h erk1/2 were upregulated, there was significantly differently between control group and uric acid treated group, Fig. 2, $p<0.05$. These results indicated the significance of MAPK signaling pathway in renal tubule epithelial cell proliferation and apoptosis regulated by uric acid. In addition, the differentially expression of NF-κB suggests the crosstalk between NF-κB signaling pathway and MAPK signaling pathway, which corresponds with the observation of Han JH et al that uric acid regulates renal tubule cell proliferation through MAPK and NF-κB signaling pathways [7].

The relationship between proteins in cell proliferation and cell apoptosis was given in Suppl. 3 and 4 (http://www.rayfile.com/zh-cn/files/06d1d14c-24a2-11e0-a784-0015c55db73d/; http://www.rayfile.com/zh-cn/files/1fb2a670-24a2-11e0-b848-0015c55db73d/) by comprehensive analysis, which also showed the importance of proteins in MAPK signaling pathway during cell apoptosis and cell proliferation.

Protein AC	Protein ID
RAC1	A4D2P0
MAP4K1	A8MWC4
NFKB2	A8K9D9
FLNB	O75369
EGFR	P00533
HSPB1	P04792
KRAS	P01116
HSPA1B	P08107
HSPA1A	P08107
STMN1	P16949
MAPK1	P28482
MAP2K3	P46734
RAP1B	P61224
CACNB2	Q08289
MAP2K1	Q02750
PAK2	Q13177
FLNA	Q60FE5
CHP	Q99653
MAPKSP1	Q9UHA4

Table 3. Proteins involved in the MAPK Signaling Pathway

Conclusion

In summary, our work gives an overview of the proteomic variations of renal tubule epithelial cells treated with elevated uric acid. In this study, LS-MS analysis was used to generate the differentially expressed protein profiles of renal tubule epithelial cells treated with elevated uric acid. This study reveals proteins and signaling pathways involved in renal tubule cell proliferation and cell apoptosis that affected by elevated uric acid. MAPK signaling pathway was shown to play a central role in this process. Proteins related with cell trans-differentiation under elevated uric acid condition were also identified in this study. In conclusion, our study has laid a solid foundation for the elucidation of the mechanisms of renal tubule cell apoptosis and interstitial fibrosis in hyperuricemia patients.

Acknowledgements

This work was supported by grant (2007CB507400) from the National Basic Research Program of China (973 Program) and grants (30670983) from the National Natural Science Foundation of China.

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