Original Paper

ALDR Enhanced Endothelial Injury in Hyperuricemia Screened using SILAC

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Key Words

Uric Acid • Endothelial Injury • ALDR • Oxidative Stress • SILAC

Abstract

Background: The exact etiology of hyperuricemia-induced endothelial injury remains ill-defined. To elucidate the mechanism that leads to endothelial injury in hyperuricemia, we investigated proteins expressed in human umbilical vein endothelial cells (HUVECs) cultured with high concentrations of uric acid (HUA) *in vitro*. **Methods:** We used stable isotope labeling with amino acids in cell culture (SILAC) combined with LC-MS/MS analysis to compare proteins expressed in HUVECs cultured in media with or without HUA. The results were confirmed by Western blotting. Reactive oxygen species (ROS) were detected using a confocal microscope. **Results:** Thirty-nine proteins with various cellular functions were differentially expressed. Among them, aldose reductase (ALDR) protein expression was enhanced significantly, indicating increased aldehyde reductase and oxidoreductase activities. ROS levels decreased when ALDR protein activity was inhibited by siALDR. **Conclusions:** ALDR protein may play an important role in endothelial injury induced by hyperuricemia, and activity of the ALDR protein is associated with oxidative stress.

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Introduction

Uric acid (UA) is the end product of purine nucleotide metabolism in humans [1-3]. Hyperuricemia results from either increased generation or decreased degradation of UA, which is biologically active and can lead to endothelial injury, oxidative stress, inflammation and vasoconstriction [4]. Hyperuricemia is an independent risk factor for many diseases, such as kidney and cardiovascular diseases [5]. Hyperuricemia contributes to endothelial dysfunction in early and middle stage chronic kidney disease (CKD), and oxidative stress is associated with this injury [5]. Previous studies have confirmed multiple mechanisms involved in endothelial dysfunction, including reduced nitric oxide (NO) bioavailability due to inhibited uptake of L-arginine, promotion of L-arginine degradation, and NO scavenging [4]. Park and colleagues suggested that high concentrations of uric acid (HUA) attenuated binding between endothelial nitric oxide synthase (eNOS) and calmodulin (CaM), consequently decreasing eNOS activity and NO production [6]. They also reported that endothelial dysfunction is related to mitochondrial changes and decreased intracellular adenosine triphosphate (ATP) [7]. Another study indicated that HUA can induce endothelial dysfunction, and this effect is associated with oxidative stress [8]. However, further studies are needed to elucidate the comprehensive mechanism leading to endothelial injury induced by HUA. Therefore, we used a new method, stable isotope labeling by amino acids in cell culture (SILAC), combined with liquid chromatograph-mass spectrometry (LC-MS), to analyze the differentially expressed proteins in human umbilical vein endothelial cells (HUVECs) cultured with HUA compared with those without UA. This new LC-MS analysis improves upon the shortcomings of previous MS analysis, and SILAC (first reported by Mann et al.) can be used to analyze differentially expressed proteins both qualitatively and quantitatively, which greatly improves the sensitivity and accuracy of measurements [9]. Gene Ontology (GO) classification and the Molecular Annotation System (MAS) were used to confirm the characteristics of these differentially expressed proteins and the interactions among them.

Here, we used the HUVEC line to screen differentially expressed proteins in cells exposed to HUA using SILAC combined with LC-MS analysis (<http://www.uniprot.org> and <http://bioinfo.capitalbio.com/mas3/> online analysis platforms).

Materials and Methods

Main reagents

SILAC™ kits and RNAi MAX transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). Trypsin and uric acid were purchased from Promega (Madison, USA). Antibodies were obtained from Santa Cruz Biotechnology (California, USA). Oxonic acid potassium salt was bought from Sigma (ST. LOUIS, USA), and the Nano-ESI LTQ-ion trap mass spectrometer was purchased from Thermo Fisher Scientific (Waltham, USA).

Cell culture

The human umbilical vein endothelial cell line (HUVEC) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was cultured in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS), 100 U/L penicillin, and 100 U/L streptomycin at 37°C under 5% CO₂. Two days prior to experiments, HUVECs were subcultured in 35 mm confocal glass bottom dishes or 25 cm² bottles, as needed.

Stable heavy isotope labeling

HUVECs were cultured in RPMI 1640 media containing 0.1 mg/mL heavy-Lys (¹³C₆-Lys) or 0.1 mg/mL light-Lys for six generations to ensure isotope labeling efficiency, according to the manufacturer's instructions (Invitrogen). The media were replenished with fresh RPMI 1640 containing 0.1 mg/mL heavy-Lys (¹³C₆-Lys) or 0.1 mg/mL light-Lys during cell passaging, and the labeling efficiency reached 99% after

six generations. Cells were seeded in 25 cm² culture flasks and classified into two groups: cells cultured in light-Lys media (non-UA) and those cultured in heavy-Lys containing HUA. Uric acid (600 µM; Sigma) was added to the media for 24 h. Cells were scraped, centrifuged, counted, and mixed at a 1:1 ratio for protein extraction.

LC-MS analysis and data processing

Extracted proteins were assessed by two-dimensional LC-MS as reported previously [9]. Data from MS analysis were searched against the NCBI Human refseq database (v.2011) using SEQUEST v.28 of Bioworks 3.31. The false discovery rate was calculated in the reverse database using trypsin as a search parameter and including up to two missed cleavage sites [9]. Initial mass deviations of precursor and fragment ions were up to 2 Da and 1 Da, respectively. Mass deviations of amino acid modifications were set to 57.02 Da and 19.02 Da for alkylated cysteine and oxidated methionine, respectively. Peptide possibility was calculated by Bioworks. The false discovery rate reached 1% after Xcorr, sp, Rsp, DeltaCn, and peptide possibility filtration. Protein coverage rate was calculated by a protein coverage summarizer. Pathway networks and protein functional grouping were generated by the online platform at <http://bioinfo.capitalbio.com/mas3.0/>.

Small interfering RNA (siRNA) transfection

ALDR siRNA (siALDR) (100 nM, sc-29204, Santa Cruz) was transfected into HUVECs using RNAi MAX transfection reagent according to the manufacturer's instructions (Invitrogen). A non-silencing siRNA was used as a negative control. Forty-eight hours after transfection, cells were starved for 16 h, followed by treatment with UA, or no treatment.

Western blot analysis

RIPA lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 1 mM PMSF, and a variety of protease inhibition agents [1 µg/mL]) was used to extract protein. Cell lysates were used to determine protein concentration using a BCA kit. Approximately 80 µg protein was loaded onto 10% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and kept overnight in 5% non-fat milk at 4°C after Ponceau S staining. The membrane was incubated in primary antibodies (Santa Cruz). Blots were developed with enhanced chemiluminescent (ECL) reagent (Santa Cruz) according to the manufacturer's instructions and exposed to X-ray film. Protein bands were quantified using quantity one software (Bio-Rad).

Establishment of hyperuricemic mice models

Hyperuricemic mice were established as described previously with slight modifications [10]. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital. Wild-type C57BL/6 mice obtained from the Experimental Animal Center of Academy of Military Medical Sciences (China) were used as controls. Mice were housed in temperature-controlled cages on a 12-h light-dark cycle and given free access to water and normal chow diet. After 1 week of breeding for adaptation, mice were grouped into control (n = 6) or hyperuricemic (n = 6) groups. Mice were injected intraperitoneally with 250 mg/Kg-d oxonic acid potassium salt (Sigma) and 250 mg/Kg-d uric acid (Sigma). Fourteen days after modeling, UA levels in the blood were evaluated, and aortas were subjected to immunohistochemistry.

Immunohistochemistry

Aortas were fixed in formalin and embedded in paraffin. Paraffin sections were cut into sections (3–4 µm thick) and mounted onto poly-L-lysine-coated slides. Endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were heated in a microwave oven for 10 min in sodium citrate buffer (pH 6.0) incubated for 20 min with 1.5% normal goat serum, followed by overnight incubation with the primary antibody (AR; dilution 1:100). As a negative control, sections were incubated with phosphate-buffered saline (PBS). After removal of unbound primary antibody, the sections were incubated for 60 min at room temperature with biotinylated secondary antibody. Sections were rinsed and incubated for 60 min with avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC Kit; Vector Laboratories). Incubation with 3,3-diaminobenzidine tetrahydrochloride as a substrate chromogen solution to produce a brown color was performed for 10 min. Finally, the sections were counterstained with hematoxylin.

Table 1. Different proteins expression in endothelial cells cultured with high UA and without UA. * ALDR expression increased in endothelial cells cultured at the presence of 600μmol/L UA for 24 h

Uniprot KB	Protein Name	Definition	Mass	NRatio
P15121	ALDR	aldo-keto reductase family 1, member B1, aldose reductase	35830	18.97
B2RA03	B2RA03	keratin, type II cytoskeletal 18	48029	1.74196
Q53HU8	Q53HU8	vimentin	53619	0.98813
Q9UII2	ATIF1	putative uncharacterized protein dkfzp564g0422	12397	0.98152
Q7L4M3	Q7L4M3	keratin, type II cytoskeletal 8	53671	0.93764
Q6IPT9	Q6IPT9	elongation factor 1-alpha 1	50109	0.90875
P08729	K2C7	keratin, type II cytoskeletal 7	51373	0.8926
Q6NUR7	Q6NUR7	ezzrin	69370	0.87473
B4DTN7	B4DTN7	isoform 1 of protein-glutamine gamma-glutamyltransferase 2	77280	0.7267
P26599	PTBP1	isoform 1 of polypyrimidine tract-binding protein 1	57186	0.72021
Q9H6X9	Q9H6X9	heat shock protein hsp 90-beta	83212	0.71646
Q86SX1	Q86SX1	isoform 2 of heat shock protein hsp 90-alpha	98099	0.6924
B4DTG2	B4DTG2	highly similar to elongation factor 1-gamma	56114	0.68625
B7Z597	B7Z597	60 kDa heat shock protein, mitochondrial	61016	0.6479
Q00610	CLH1	isoform 1 of clathrin heavy chain 1	191493	0.61413
P68366	TBA4A	tubulin alpha-4a chain	49892	0.577
P22626	ROA2	isoform b1 of heterogeneous nuclear ribonucleoproteins a2/b1	37407	0.57486
D2JYH4	D2JYH4	actin, aortic smooth muscle	41982	0.56132
B2RDW1	B2RDW1	ubiquitin and ribosomal protein s27a precursor	17953	0.54504
Q8TES4	Q8TES4	isoform 2 of filamin-a	279843	0.52057
P12814	ACTN1	alpha-actinin-1	102993	0.501
Q53GK6	Q53GK6	actin, cytoplasmic 1	41710	0.4983
B3KUK2	B3KUK2	superoxide dismutase [MN], mitochondrial	24707	0.4851
O75369	FLNB	isoform 1 of filamin-b	278021	0.48375
Q5TZZ9	Q5TZZ9	annexin a1	38690	0.44331
O43707	ACTN4	alpha-actinin-4	104788	0.4385
Q96HX0	Q96HX0	tubulin beta-2c chain	49799	0.4044
P14618	KPYM	isoform m2 of pyruvate kinase isozymes m1/m2	57900	0.3754
Q0QET7	Q0QET7	glyceraldehyde-3-phosphate dehydrogenase	36030	0.37292
A4UCS8	A4UCS8	isoform alpha-enolase of alpha-enolase	47139	0.3717
Q13509	TBB3	tubulin beta-3 chain	50400	0.33322
P00338	LDHA	isoform 1 of l-lactate dehydrogenase a chain	36665	0.33172
P11021	GRP78	hspa5 protein	72377	0.30588
B7Z4V1	B7Z4V1	alpha-actinin-2	103788	0.2234
P07355	ANXA2	isoform 2 of annexin a2	40386	0.2198
A6NMY6	AXA2L	putative annexin a2-like protein	38635	0.21773
Q1ZYQ1	Q1ZYQ1	isoform 1 of tubulin alpha-3c/d chain	49928	0.1477
B4DK16	B4DK16	pre-mrna-processing-splicing factor 8	273427	0.13834
B4DM32	B4DM32	translational activator GCN1	292558	0.02688

Measurement of total intracellular ROS

Changes in intracellular ROS levels were detected using the oxidant-sensing fluorescent probe CM-H₂DCFDA [11]. This probe was loaded into previously subcultured HUVECs at a final concentration of 10 μmol/L, and cultured at 37°C for 30 min in the dark. After incubation, the culture media were washed twice with PBS (10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) to remove the dyes from the media, and analyzed using laser confocal microscopy. Fluorescence images were collected with a laser confocal system mounted onto an inverted microscope equipped with an argon-krypton laser. For studies with CM-H₂DCFDA, an excitation wavelength of 488 nm (argon laser) and emission wavelength of 515 nm were used.

Statistical analysis

Results are presented as the means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) plus Bonferroni's correction (Student-Newman-Keuls). A P value less than 0.05 was considered statistically significant.

Fig. 1. GO analysis of all differentially expressed proteins. (A) Thirty-two proteins are involved in cellular component, 34 in molecular function, and 31 in biological processes. (B) Protein numbers in the molecular function subgroup. (C) Protein numbers in the cellular components subgroup. (D) Protein numbers in the biological processes subgroup.

Results

LC-MS analysis

There were 39 differentially expressed proteins with an Nratio (the change in the ratio of the up/downregulated proteins induced by uric acid relative to the control) range of $20 > \text{Nratio} > 1.5$ (upregulation) or $0.05 < \text{Nratio} < 0.66$ (downregulation). Table 1 shows the differentially expressed proteins.

Gene Ontology analysis of differentially expressed proteins

Differentially expressed proteins were analyzed according to Gene Ontology (GO) classification. Among these proteins, 32 are associated with cellular component, 34 with molecular function, and 31 with biological processes (Fig. 1A). In the molecular function category, 25 proteins are related to binding, while the others are related to catalytic or

Table 2. 21 proteins are classified to cellular metabolic process

Uniprot KB	Protein Name	Definition
P15121	ALDR	Aldose reductase, aldo-keto reductase family 1, member B1
Q9UII2	ATIF1	ATPase inhibitor, mitochondrial
Q00610	CLH1	Clathrin heavy chain 1
P11021	GRP78	78 kDa glucose-regulated protein
P14618	KPYM	Pyruvate kinase PKM
P00338	LDHA	L-lactate dehydrogenase A chain
P26599	PTBP1	Polypyrimidine tract-binding protein 1
P22626	ROA2	Heterogeneous nuclear ribonucleoproteins A2/B...
P68366	TBA4A	Tubulin alpha-4A chain
Q13509	TBB3	Tubulin beta-3 chain
B4DTG2	B4DTG2	Elongation factor 1-gamma
B2RDW1	B2RDW1	Ribosomal protein S27a, isoform CRA_c
Q1ZYQ1	Q1ZYQ1	Tubulin, alpha 2
B3KUK2	B3KUK2	Superoxide dismutase
Q86SX1	Q86SX1	Full-length cDNA 5-PRIME end of clone CS0DN00...
Q96HX0	Q96HX0	TUBB2C protein
Q6IPT9	Q6IPT9	Elongation factor 1-alpha
B4DTN7	B4DTN7	cDNA FLJ57198, highly similar to Protein-glut...
B7Z597	B7Z597	cDNA FLJ54373, highly similar to 60 kDa heat ...
Q9H6X9	Q9H6X9	cDNA: FLJ21717 fis, clone COL10322
A4UCS8	A4UCS8	Enolase

structural molecular function (Fig. 1B). Almost all proteins are related to cell components that determine cell parts, organelles, etc. (Fig. 1C). In the biological process category, 28 proteins are related to cellular processes (Fig. 1D). In this subgroup, 21 proteins are involved in cellular metabolic processes (Table 2). Other biological processes include those of multicellular organisms, e.g., biological regulation and response to stimuli.

Pathway analysis

Pathway networks involving the differentially expressed proteins were generated using the MAS system (Table 3 and <http://yunpan.cn/QpHqtKHsHZMV4>). The prominent pathways were those involving EHEC, focal adhesion, gap junctions, leukocyte transendothelial migration, pyruvate metabolism, regulation of actin cytoskeleton, and others. The main proteins involved in these pathways included EZR, TUBA3C, ACTB, TUBB3, TUBB2C, TUBA4A, FLNA, FLNB, ACTN4, ACTN1, among others.

Analyses of ALDR protein

The ALDR protein, encoded by the aldo-keto reductase family 1 member B1 (*AKR1B1*) gene, was upregulated ~18-fold when cells were stimulated with HUA (Table 1). Therefore, we focused on this protein and analyzed the protein-GO networks of all proteins to discover the function of ALDR. The supplementary materials in this paper depict the protein-GO networks of all proteins. The protein-GO networks and pathways related to ALDR indicated that its functions include aldehyde reductase activity, oxidoreductase activity, and electron carrier activity (Fig. 2A), and that it is involved in oxidation reduction, the response to stress or other biological processes, pentose/glucuronate interconversions, and fructose/mannose metabolism or other pathways (Fig. 2B). It is also located in many cellular component (Fig. 2C). The details are listed in Table 4.

Table 3. Kegg Pathway Index

Pathway	Count	p-Value	q-Value	Gene
Pathogenic Escherichia coli infection - EHEC	6	6.56E-13	8.75E-13	EZR;TUBA3C;ACTB;TUBB3;TUBB2C;TUBA4A
Focal adhesion	5	1.27E-07	4.63E-08	FLNA;FLNB;ACTB;ACTN4;ACTN1
Gap junction	4	2.70E-07	7.70E-08	TUBA3C;TUBB3;TUBB2C;TUBA4A
Leukocyte transendothelial migration	4	7.76E-07	1.94E-07	EZR;ACTB;ACTN4;ACTN1
Regulation of actin cytoskeleton	4	8.04E-06	1.07E-06	EZR;ACTB;ACTN4;ACTN1
Pyruvate metabolism	3	1.92E-06	3.84E-07	PKM2;AKR1B1;LDHA
Glycolysis / Gluconeogenesis	3	7.43E-06	1.06E-06	PKM2;GAPDH;LDHA
Adherens junction	3	1.40E-05	1.72E-06	ACTB;ACTN4;ACTN1
Antigen processing and presentation	3	2.00E-05	2.00E-06	HSP90AB1;HSP90AA1;HSPA5
Tight junction	3	7.11E-05	5.17E-06	ACTB;ACTN4;ACTN1
Prostate cancer	2	0.001241361	6.37E-05	HSP90AB1;HSP90AA1
Systemic lupus erythematosus	2	0.003271697	1.49E-04	ACTN4;ACTN1
MAPK signaling pathway	2	0.011417746	3.46E-04	FLNA;FLNB
Cysteine metabolism	1	0.007636706	2.63E-04	LDHA
Pentose and glucuronate interconversions	1	0.014636105	4.30E-04	AKR1B1
Galactose metabolism	1	0.015217238	4.41E-04	AKR1B1
Propanoate metabolism	1	0.019854431	5.59E-04	LDHA
Fructose and mannose metabolism	1	0.021010439	5.84E-04	AKR1B1
Type II diabetes mellitus	1	0.026196251	6.99E-04	PKM2
Glycerolipid metabolism	1	0.027345056	7.20E-04	AKR1B1
Cholera - Infection	1	0.034210528	8.66E-04	ACTB
Insulin signaling pathway	1	0.078257149	0.001537602	PKM2
Purine metabolism	1	0.085858363	0.001619955	PKM2
Alzheimer's disease	1	0.099282077	0.001772894	GAPDH

Fig. 2. Protein-GO networks and interactions with the ALDR (AKR1B1) protein. (A) Significant molecular function network of the ALDR protein. (B) Significant cellular structure network of the ALDR protein. (C) Significant biological process network of the ALDR protein.

Table 4. GO and pathway analysis of ALDR

Molecular Function	Biological Process	Cellular Component	Pathway
GO:0004032 aldehyde reductase activity	GO:0005975 carbohydrate metabolism	GO:0005615 extracellular space	Pentose and glucuronate interconversions
GO:0005515 protein binding	GO:0006950 response to stress	GO:0005737 cytoplasm	Fructose and mannose metabolism
GO:0009055 electron carrier activity	GO:0055114 oxidation reduction	GO:0005829 cytosol	Galactose metabolism
GO:0016491 oxidoreductase activity			Glycerolipid metabolism
			Pyruvate metabolism

Table 5. Diseases related with ALDR. OMIM: Online Mendelian Inheritance in Man (OMIM database) ; GAD: The Genetic Association Database

Name	Source
OMIM 103880	OMIM
OMIM 103188	OMIM
nephropathy in other diseases	GAD
hypospadias	GAD
microangiopathy	GAD
neuropathy, diabetic	GAD
microvascular complications	GAD
diabetes, type 1	GAD
diabetic glomerulopathy; glomerulopathy, diabetic; kidney failure;	GAD
retinopathy, diabetic	GAD
nephropathy, diabetic; retinopathy, diabetic	GAD
diabetes, type 2	GAD
Type 2 diabetes	GAD

Combining these results, we analyzed the interacting proteins and diseases related to ALDR using MAS. The ALDR protein interacted with many other proteins (see <http://yunpan.cn/QpHqtKHsHZMV4>), including IKBKE, ZNF253, CDC, and others. Diseases related to ALDR are listed in Table 5, including nephropathy, diabetes, microvascular complications, and retinopathies. This suggests that the ALDR protein plays an important role in the vascular disease process.

ALDR in a hyperuricemia mouse model and in HUVECs

Sera derived from hyperuricemic mice and normal control mice were used to detect levels of UA, while aortas were used for immunohistochemistry. The results indicate that serum UA levels were clearly higher in the hyperuricemic mice than in the control group (Fig. 3A). Expression of ALDR was upregulated in the aortas of the hyperuricemic mice compared with the normal controls (Fig. 3B). The cell extract from HUVECs was subjected to Western blotting to determine ALDR protein expression, which was significantly enhanced in HUVECs cultured with HUA (Fig. 3C).

ROS production in endothelial cells decreased after ALDR inhibition

It was confirmed that HUA can lead to ALDR protein overexpression in HUVECs *in vitro*. HUVECs were transfected with siALDR to inhibit ALDR. To examine ALDR protein expression, the extract was examined using Western blot analysis. Overexpression of ALDR was inhibited (Fig. 4A), indicating that siALDR effectively inhibited ALDR protein expression.

Our recent research has demonstrated that HUA induces endothelial injury via ROS production. We used siALDR to downregulate the ALDR protein and subsequently measured ROS levels. The results show that intracellular ROS levels were lower in HUVECs transfected with siALDR and incubated with HUA than in non-siRNA transfection cells (Fig. 4B).

Fig. 3. ALDR protein expression *in vivo* and *in vitro*. (A) Hyperuricemic mice were injected intraperitoneally with oxonic acid potassium salt and UA for 14 days; serum UA levels increased significantly ($*p < 0.05$ compared with controls, $n = 6$). (B) ALDR protein expression was enhanced in mouse aortas, as determined by immunohistochemistry ($\times 200$). (C) ALDR protein expression increased in HUVECs cultured with $600 \mu\text{M}$ UA for 24 h ($*p < 0.05$ vs. control, $n = 6$).

Fig. 4. ALDR protein expression and ROS levels in HUVECs after transfection with siRNA. (A) ALDR protein expression was decreased in HUVECs cultured in medium containing $600 \mu\text{M}$ UA after transfection with siALDR to inhibit the ALDR protein ($*p < 0.05$ vs. siCon group, $n = 6$). (B) HUVECs were transfected with siALDR and treated with HUA for 24 h; ROS production decreased compared with the non-siRNA transfection group ($*p < 0.05$ vs. siCon group, $n = 6$).

Discussion

The number of hyperuricemia cases is increasing worldwide. Hyperuricemia has been confirmed as an independent risk factor for CKD and cardiovascular disease, among other ailments [12, 13]. Hyperuricemia induces endothelial injury, but the mechanism has not

been well investigated [14]. Several reports have analyzed differentially expressed proteins in the development of hyperuricemia. Our objective was to identify the differentially expressed proteins in HUVECs cultured with HUA. Therefore, we analyzed differentially expressed protein profiles using SILAC and LC-MS/MS. Proteomics applied on a large scale may provide useful information. The results identified 39 differentially expressed proteins among three GO groups, 32 of which contributed to cellular component, 34 to molecular function, and 31 to biological processes, particularly ALDR protein upregulation. ALDR is an aldehyde-metabolizing enzyme, a member of the aldo-keto reductase superfamily, and a regulator of ROS signaling [15]. Previous studies have confirmed that the ALDR protein plays a key role in various diseases, such as diabetic nephropathy and gastrointestinal neoplasia [15-17]. Based on the above results, we analyzed the protein-GO networks of all proteins and found that ALDR possesses aldehyde reductase, oxidoreductase, electron carrier, and other activities. Changes in ALDR are related to endothelial cell injury in uremia [18], and this function is associated with ROS, which can be generated by several mechanisms [13, 19]. Activation of the polyol pathway has been observed in diabetes and can lead to diabetic microvascular dysfunction via oxidative stress [20]. Expression of ALDR protein and mRNA is upregulated in diabetic rats compared with normal controls; superoxide anions and malondialdehyde (MDA) were increased concomitantly, while superoxide dismutase (SOD) was decreased in cultured mesangial cells treated with high glucose; inhibition of ALDR and reduced oxidative stress ameliorated renal injury [21, 22]. We also detected upregulation of the ALDR protein in other diseases, including hyperlipidemia and atherosclerosis [23]. Researchers have confirmed that transforming growth factor- β 1 (TGF- β 1) induces upregulation of ALDR through activation of ROS and the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway in human renal mesangial cells (HRMCs) [24]. High glucose leads to ALDR overexpression by increasing NF- κ B binding activity, and silencing the NF- κ B genes can reverse ALDR overexpression [25]. Some studies have also found that the expression of ALDR is mediated by TonEBP to maintain hypertonicity in the kidney medulla [26, 27]. These proteins were not found in the 39 differentially expressed proteins in our experiment and were not significantly differentially expressed in the HUA group compared with the control group (data not shown), but this result may be limited by the precision and sensitivity of the detection method. No research has examined the relationship between HUA and ALDR, and this is the first report that ALDR levels are upregulated in HUVECs cultured with HUA. The mechanism by which HUA induces overexpression of ALDR is unknown, but we hypothesize that HUA increases ALDR by altering normal physiological functions. Understanding the exact mechanism by which HUA induces ALDR overexpression will require further studies. NADPH is a co-factor for the ALDR protein and a substrate in the generation of GSH; the ALDR protein depletes NADPH and consequently decreases GSH, such that the antioxidant capacity is impaired by the increased levels of ALDR protein [20, 28]. Based on these results, we propose a relationship between ALDR and ROS. Also, intracellular levels of ROS blocked by siALDR were lower than those of the non-siRNA transfection group. We speculate that the ALDR protein plays an important role in endothelial injury caused by hyperuricemia via oxidative stress. However, the precise mechanism remains unclear; thus, further studies are needed to explain the relationship between ALDR and endothelial injury.

Conclusion

Our work provides an overview of the proteomic variations in HUVECs treated with HUA. In this study, SILAC combined with LC-MS/MS analysis was used to determine the differentially expressed protein profiles in HUVECs treated with HUA. We found that expression of the ALDR protein increased significantly and that ROS levels were related to ALDR protein levels. This study also revealed that ALDR plays a vital role in endothelial injury and oxidative stress induced by HUA. Thus, our results may facilitate a more complete understanding of the mechanism behind endothelial injury induced by HUA.

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