International Journal of Cardiology 176 (2014) 746–752

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

A global proteome approach in uric acid stimulated human aortic endothelial cells revealed regulation of multiple major cellular pathways

Andreas Oberbach [a,b], Jochen Neuhaus [c], Nico Jehmlich [d,e], Nadine Schlichting [f], Marco Heinrich [a], Yvonne Kullnick [a], Friedrich-Wilhelm Mohr [a], Joachim Kugler [b], Sven Baumann [g], Uwe Völker [d], Volker Adams [h,*]

[a] Department of Cardiac Surgery, Heart Center Leipzig, University of Leipzig, Leipzig, Germany
[b] Department of Health Sciences/Public Health, University of Dresden, Dresden, Germany
[c] Department of Urology, University of Leipzig, Leipzig, Germany
[d] Ernst-Moritz-Arndt-University Greifswald, Interfaculty Institute of Genetics and Functional Genomics, Department of Functional Genomics, Germany
[e] Helmholtz Centre for Environmental Research, Department of Proteomics, Leipzig, Germany
[f] Department of Pediatric Surgery, University of Leipzig, Leipzig, Germany
[g] Helmholtz Centre for Environmental Research, Department of Metabolomics, Leipzig, Germany
[h] Department of Cardiology, University of Leipzig, Heart Center Leipzig, Leipzig, Germany

ARTICLE INFO

Article history:
Received 21 March 2014
Received in revised form 1 July 2014
Accepted 26 July 2014
Available online 4 August 2014

Keywords:
Cardiovascular disease
Uric acid
Ubiquitin–proteasome pathway
Eukaryotic initiation factor 4F (eIF4)
Hypoxia-inducible factor 1-alpha (HIF-1α)
Global proteome approach (2D-nano LC–MS/MS)

ABSTRACT

Background: Uric acid (UA) has been identified as one major risk factor for cardiovascular diseases. Lowering of serum UA levels improves endothelial function. The present study investigates for the first time concentration-dependent effects of UA on human aortic endothelial cells (HAEC) and the cellular pathways involved in global proteomic analysis.
Methods: The concentration dependent effects of UA on HAEC were investigated by nanoLC–MS/MS and ingenuity pathway analysis to reveal putative cellular pathways. For verification of the identified pathways the abundance or activity of key proteins was measured using ELISA or Western blotting. NO production was quantified by confocal laser microscopy.
Results: We identified ubiquitin–proteasome system (UPS) and eIF4 signaling as the major pathways regulated by UA. K-means clustering analysis revealed 11 additional pathways, of which NO, superoxide signaling and hypoxia were further analyzed. A complex regulatory network was detected demonstrating that 500 μmol/L UA, which is well above the concentration regarded as pathological in clinical settings, led to diminishing of NO bioavailability. In addition a UA-dependent downregulation of eIF4, an upregulation of UPS and an increase in HIF-1α were detected.
Conclusions: Here we show for the first time, that increasing UA levels activate different sets of proteins representing specific cellular pathways important for endothelial function. This indicates that UA may alter far more pathways in HAEC than previously assumed. This regulation occurs in a complex manner depending on UA concentration. Further studies in knockout and overexpression models of the identified proteins are necessary to prove the correlation with endothelial dysfunction.

1. Introduction

Uric acid (UA) is the end product of the purine metabolism, and is implicated in many disease conditions. Although UA has useful antioxidant properties at the cellular level [1,2] sustained hyperuricemia has putative adverse effects in cardiovascular diseases. Epidemiological data support that increased serum UA is a relevant independent risk factor for cardiovascular and renal disease, especially in patients with hypertension, heart failure and diabetes [3]. In addition an increased level of UA is an independent predictor for mortality in coronary heart disease, heart failure and stroke [4–6].

The circulating concentration of UA is a function of the balance between the breakdown of purines in several tissues and the rate of serum UA (sUA) excretion [7] by the kidneys and elimination via the intestinal tract [8]. One has to be aware that chronic elevated circulating UA will affect multiple organs either directly or via impairment of endothelial function [9].

* Corresponding author at: Department of Cardiology, University of Leipzig, Heart Center Leipzig, Struempellstr 39, 04289 Leipzig, Germany. Tel.: +49 341 865 1671; fax: +49 341 865 1461.
E-mail address: adav@medizin.uni-leipzig.de (V. Adams).

http://dx.doi.org/10.1016/j.ijcard.2014.07.102
0167-5273/

There is growing evidence that lowering uric acid with specific xanthine-oxidase inhibitors improves endothelial dysfunction in different diseases [10,11]. In detail, UA influences cellular metabolism, especially nitric oxide (NO) signaling and production of reactive oxygen species (ROS), which play a major role in the regulation of cellular function. Recently, Park et al. reported that one possible mechanism how UA decreases NO production is by reducing the interaction of endothelial nitric oxide synthase (eNOS) and calmodulin, an eNOS activator [12]. In addition scavenging NO by ROS may be another mechanism of lowering NO bioavailability [13]. It is known that UA resulted in vascular smooth muscle cell proliferation by increasing platelet derived growth factor [14] and hypertrophy via vascular renin–angiotensin system [15,16], both are major events in atherosclerosis etiology.

From the clinical perspective, elevated serum UA levels are associated with an increased risk of several diseases [17,18] and this concentration dependency is also reflected in different cellular responses as reported in numerous cell culture studies [16,19].

These findings support the hypothesis that UA may promote a variety of signaling pathways in a concentration-dependent way. To investigate those probably complex cellular metabolic processes a more global approach is needed to perform a holistic evaluation and reveal deeper insight into UA-induced changes. Since elevated circulating UA directly influences the cellular physiology of endothelial cells we investigated cultured human aortic endothelial cells (HAEC) in order to describe the concentration-dependent effects of UA.

2. Material and methods

2.1. Cell culture

The study design is shown in Fig. S1. HAEC (Cell Systems Biotechnology, Troisdorf, Germany) were cultured in EGM-2 cell culture medium (Lonza, Walkersville MD, USA) until 80 to 90% confluence. Cells were incubated with UA (100, 300, 500 μmol/L) for 24 h and harvested either with ice cold lysis buffer (50 mmol/L Tris–HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Triton X-100, 0.2% SDS) containing protease inhibitor mix M (Serva, Heidelberg, Germany) for Western blot analysis or 8 mol/L urea/2 mol/L thiourea for proteome analysis. Protein concentration was determined using BSA as standard (BCA method, Pierce, Rockford IL, USA). For inhibition of proteasome activity HAEC were pre-incubated for 1 h with 100 nmol/L MG132 (Calbiochem (EMD Millipore), Darmstadt, Germany), followed by additional incubation with UA (100, 300, 500 μmol/L) or medium with MG132 (100 nmol/L) for 24 h.

2.2. Mass spectrometric analysis

Protein lysates of HAEC after the UA incubation were analyzed by LC–MS as described earlier [20]. Tryptically cleaved peptides (500 ng) were separated prior to MS analyses by reverse phase nano HPLC on a 15 cm Acclaim PepMap100-column (C18, 3 μmol/L, 100 Å) using an EASY-nLC system (Proxeon, Thermo Scientific, Waltham, MA, USA) at a constant flow rate of 300 nL/min. Separation was achieved using a non-linear gradient of 70 min with 0.1% acetic acid, 2% acetonitrile in water (solvent A) and 0.1% acetic acid in 100% acetonitrile (solvent B). Separated peptides were analyzed by an LTQ Orbitrap Velos MS (Thermo Scientific). The MS-instrument was operated in data-dependent acquisition (DDA) mode. MS/MS scan events were repeated for top 12 peaks using the higher energy dissociation mode (HCD) at normalized collision induced energy of 35%, activation time of 100 ms, and minimum of ion signal threshold for MS/MS of 2000 counts.

Raw data were processed using the Refiner MS 7.5 and Analyst 7.5 module (Genedata, Basel, Switzerland). Refiner MS performed peak detection, noise analysis, peak integration, isotope grouping and retention time alignment of the acquired MS data. Generated peak lists were searched against a human Uniprot/Swiss-Prot database (containing 20,268 unique entries, release 2011_10) using an in-house Mascot server 2.3.2 (Matrix Science, London, GB). Searches were performed with carbamidomethyl on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion tolerance (MS) and 20 mmu MS/MS tolerance. Only ranked one peptide hits with a Mascot ion score >23 were considered as identified. After peak annotation, the data were processed in Analyst 7.5 (Genedata), where statistical data evaluation was performed using univariate methods. ANOVA (analysis of variance) was selected to compute the significance of differential protein expression in one group and represents an extension of the T-test to $k > 2$ groups. A parametric T-test was used for the two group comparison and a p-value of less than 0.05 was considered as statistically significant. Proteins were functionally assigned to gene ontology terms and canonical pathway analysis was performed using ingenuity pathway analysis (v14197757, Ingenuity Systems, Redwood City, CA, USA).

2.3. Detection of NO production in human aortic endothelial cells

HAEC were incubated with increasing concentrations of UA (0, 300, 500 μmol/L) for 24 h. After a first wash with HBSS (Biochrom AG, Berlin, Germany), cells were incubated with 5 μmol/L 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate dissolved in HBSS for 30 min at 37 °C in the dark and NO production was quantified as described elsewhere [21,22] using a LSM5 Pascal laser scanning microscope (Zeiss, Jena, Germany).

2.4. Detection and quantification of ROS

HAEC were incubated with increasing concentrations of UA (100, 300 and 500 μmol/L) for 24 h. To quantify ROS production by flow cytometry, adherent cells were stained with CellRox deep red reagent (Molecular Probes, Leiden, The Netherlands) for 30 min at 37 °C in the dark. Cells were washed 2 times with phosphate buffer solution before the detached cells were analyzed within 60 min by flow cytometry (LSR II, Becton Dickinson, Heidelberg, Germany). For quantitative analysis gates were placed according to the negative control (0 μM UA) and the amount of cells in this gate was evaluated for all UA concentrations.

2.5. Validation of UA dependent pathways in HAEC

Ten micrograms of HAEC total protein was separated on a denaturing polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The transferred proteins were incubated with 1:200 dilution of anti-eIF4E, anti-NOS3, anti-HIF-1α antibody (all Santa Cruz, Heidelberg, Germany), anti-Akt, anti-phospho-Akt (both from Cell Signaling Technology, Leiden, The Netherlands), or anti-phospho-eNOS-Ser1177 (Becton Dickinson, Heidelberg, Germany) at 4 °C overnight. The bound antibodies were detected by a peroxidase coupled anti rabbit or anti mouse antibody (Cell signaling, Boston, MA USA, 1:1000 for 1 h RT) followed by a chemiluminescent reaction using luminol (SuperSignal West Pico, Pierce Rockford, Il, USA). To quantify protein expression the blots were analyzed by densitometry with a 1-D analysis software package (One-Dscan, Scanalytics, Billerica, MA, USA). To control for loading differences, the blots were also probed with an antibody against GAPDH (1:30.000; Hytest, Turku, Finland). The densitometry results were expressed as ratio between target protein and GAPDH intensity.

Total ubiquitin, total poly-ubiquitinated proteins and 20S proteasome activity were determined by commercial kits according to the manufacturer's instruction (UbiQuant quantitative ubiquitin ELISA, LifeSensors, Malvern, PA, USA; poly-ubiquitinated protein ELISA Kit, CycLex, Nagano, Japan; 20S Proteasome Activity Assay Kit, Chemicon (EMD Millipore)).

The activity of antioxidants like catalase (CAT), xanthinoxidase (XO) and glutathione peroxidase (GPX) was measured by enzyme activity assays (BioVision, Milpitas, CA, USA).

The concentration of tyrosine nitration in HAEC lysates was analyzed by OxiSelect Nitrotyrosine ELISA Kit (Cell Biolabs, San Diego, CA, USA).

2.6. Statistical analysis

As recently published data analysis was performed using Prism v6.0 (GraphPad Software, La Jolla, USA) [23]. Statistical differences were calculated by ANOVA or independent Student t-test. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Proteome analysis of HAEC after UA stimulation

We investigated concentration-dependent protein expression induced by UA in HAEC using proteomics (Fig. 1). In total 2736 unique annotated peptides which were assigned to 771 distinct proteins (420 proteins with ≥2 peptides per protein) were identified (Table S1). Fig. 1A shows the 125 proteins significantly regulated by distinct UA concentrations and most changes of protein expression were found comparing medium control versus 300 μmol/L UA ($n = 62$ proteins). Only 4 proteins are commonly regulated at all three UA concentrations (Fig. 1A).

A partial least square (PLS) analysis was performed to explain the maximum multidimensional variance of 771 identified proteins in response to UA stimulation (Fig. 1B). In detail, 300 μmol/L UA samples clearly showed poor correlation within the sample group indicating a possible threshold concentration of UA stimulation on HAEC. Out of the 420 identified proteins, 125 proteins were significantly different on the basis of an analysis of variance ANOVA test ($p \leq 0.05$) (Table S2; Fig. 1B). Enrichment analysis of proteomic data (differential proteins $n = 125$) was performed by ingenuity pathway analysis (IPA). Each enriched category is assigned an adjusted p-value (Fisher's exact test) and displays the most significant canonical pathways across