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Effect of 21-day head down bed rest on urine proteins related to endothelium: Correlations with changes in carbohydrate metabolism

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ABSTRACT

We performed liquid chromatography-mass spectrometric study of the urine proteome in 8 healthy volunteers aged between 20 and 44 y.o. who have completed 21-day head-down bed rest. ANDSystem software which builds associative networks was used to identify the urinary proteins functionally related to the endothelium. We identified 7 endothelium-related biological processes, directly linked to 13 urine proteins. We performed manual annotation of the proteins which were the most important in terms of endothelial functions.

Analysis of the correlations with biochemical variables revealed a positive correlation between fasting blood glucose and the following urine proteins: albumin, CD44 antigen, endothelial protein C receptor, mucin-1, osteopontin, receptor tyrosine kinase. As well, we found a positive correlation between HOMA-insulin resistance index and the following urine proteins: endothelial protein C receptor and syndecan-4. These results might suggest the involvement of above-mentioned proteins in glucose metabolism and their participation in the response to changes in blood glucose level.

1. Introduction

Head-down bed rest (HDBR) is a strict bed rest with a negative −6° tilt. HDBR is widely used to simulate the effects of microgravity on various physiological systems, especially for musculoskeletal and cardiovascular studies. HDBR is characterized by an enhanced physical inactivity and an upward fluid shift [1], resulting in various alterations in the different body systems. HDBR triggers adaptive mechanisms of cardiovascular, endocrine, central and peripheral nervous systems [2,3]. With regard to cardiovascular system, HDBR induces hypovolemia and cardiovascular deconditioning with alteration in vascular functions of various body regions [4]. Cardiovascular deconditioning is accompanied by endothelial dysfunction which affects numerous body processes, including functioning of blood-organ barriers [5].

Simulated microgravity was shown to markedly modulate the expression of individual genes in endothelial cells [6]. In human umbilical vein endothelial cells (HUVEC) secretome in vitro, microgravity simulated by random positioning machine induced a decrease in the proangiogenic factor FGF-2 and pro-inflammatory cytokines IL-1 and IL-8, while chemokines involved in leukocyte recruiting, RANTES and eotaxin, were increased [6]. Gene expression profile of HUVEC cultured on the ISS for 10 days showed a modulation of 1023 genes, mostly involved in cell adhesion, oxidative phosphorylation, stress response, cell cycle regulation and apoptosis, with the most of up-regulation occurring for the thioredoxin-interacting proteins. It is suggested that microgravity creates a prooxidant environment in HUVEC culture, that alters endothelial function and promotes aging [7,8]. It is known that endothelial dysfunction is the first step in the development of atherosclerosis associated with insulin resistance syndrome. Therefore, the search for countermeasures of endothelial dysfunction remains the most promising pathway for prevention and treatment of atherosclerosis. All conditions included in metabolic syndrome constellation (hyperglycemia, hypertension, hypercholesterolemia) enhance endothelial dysfunction. In case of a primitive endotheliopathy, transendothelial insulin transport would be altered with an eventual development of insulin resistance. Such insulin resistance would be secondary to endotheliopathy [9].

Increase in blood insulin level following spaceflight was demonstrated in animals as well as in human astronauts [10,11]. Such an increase was also observed following various durations of HDBR. It may be related to islet remodeling or to changes in carbohydrate metabolism under simulated microgravity [12]. It is known that blood

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glucose level remains normal during physical inactivity (due to increase in beta-cell mass and function). Insulin acts on carbohydrate metabolism promoting glucose uptake and utilization. It contributes to glucose conversion into glycogen in the liver. Insulin also inhibits endogenous glucose production by suppressing glycogenolysis and gluconeogenesis [13]. Insulin acts via transmembrane tyrosine kinase receptors, which provide a phosphorylation of specific intracellular proteins – insulin receptor substrates. It has been shown that even physiological concentrations of insulin might contribute to the shift of vascular endothelial phenotype into pathology in cells previously damaged by exposure to high glucose levels [14]. In the study of Mikhailov et al. on exercise tolerance in healthy volunteers before and after 120-day HDBR, baseline blood lactate, free fatty acids, total lipids and lactate dehydrogenase activity differed significantly before and after HDBR. The most informative variables for evaluation of individual reaction appeared to be blood insulin, cortisol, triglycerides, glucose and malate dehydrogenase activity [2].

Endothelial functions are mainly evaluated using the instrumental tests, such as flow-mediated dilation, evaluation of reactive hyperemia, pulse wave analysis, laser Doppler flowmetry of the skin, response to pharmacological or physical stimuli, and others. Blood and urine studies also contribute to the assessment of endothelial dysfunction. The existing laboratory methods allow for assessment of many of the substances produced by the endothelium in blood and urine samples [15]. Owing to its availability, easiness of collection and correlation with physiological or pathophysiological changes, urine has been the sample of choice in the majority of clinical proteomics studies, particularly those addressing renal diseases. So we suggest that study of the urine protein composition during HDBR could reveal new data about the physiological changes observed in bed rest.

The aims of the present work were to identify urine proteins related to endothelium and to reveal the relationships between these proteins and biochemical variables related to carbohydrate metabolism and its regulation.

2. Materials and methods

2.1. 21-day bed rest study

Mass spectrometry-based proteomics was employed to analyze urine samples from 8 healthy volunteers aged between 20 and 44 years old. Volunteers remained at −6° HDBR without countermeasures for 21 days, under continuous supervision. The study was performed at the facilities of the French Institute for Space Medicine and Physiology (MEDES, Toulouse, France). The subjects received a standard diet according to the recommendations of the World Health Organization. All nutrients and water consumption was controlled throughout the study. Urine was collected at the baseline (7 days before HDBR) and at the end of HDBR (day 21of HDBR), by free urination.

All procedures and risks associated with the experiments were explained, and written informed consent was obtained from each participant. The experimental protocol conformed to the Helsinki Declaration and was approved by Toulouse ethical committee.

2.2. Urine samples preparation and analysis

The sample collection methodology (samples processing, storage, *etc.*) was identical before and at the end of HDBR. Samples were collected midstream from the second urine void in the morning and then stored immediately at 4 °C. Then urine samples were centrifuged at 2000*g* for 10 min at 0–4 °C to remove cell debris and the supernatant was frozen at −80 °C until analysis. Urine samples were concentrated using Amicon Ultra Ultracel 3 k tube (Millipore, USA) at 1000g for 1 h at 4 °C. The resultant concentrate was then evaporated to dryness. Afterwards samples were modified for mass spectrometry analysis, which contains reduction, alkylation, proteins precipitation and digest by trypsin [16].

Gained polypeptide chains were divided by liquid chromatography via nano-HPLC Agilent 1100 system (Agilent Technologies, Santa Clara, CA) in combination with a 7-Tesla LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanospray ion source. Liquid chromatography- mass spectrometry data were searched using a Mascot software search engine (Matrix Science, London, UK; version 2.0.04) to identify proteins from the human International Protein Index (IPI) protein sequence database from the European Bioinformatics Institute (version 3.82; released 06.04.2011; 92,104 entries).

2.3. Bioinformatic and statistical analysis

Associative networks describing interaction between revealed proteins were constructed using automatic data extraction software ANDSystem [17]. Associative networks included all the proteins found in the urine proteome of healthy volunteers. Links of these proteins with functional state of endothelium were established through the identifier *endothel*. This identifier corresponded to 190 biological processes, according to the gene ontology specified in GO database and available in the knowledge base of ANDCell system.

Correlation analysis was performed using Statistica 10 software. Correlation matrix based on Pearson correlation coefficient with a significance level of 0.05. F-test for homogeneity of variances of samples was performed with one-way ANOVA.

2.4. Blood studies

Antecubital venous blood samples were collected 7 days before HDBR and at the end of HDBR (day 21). Blood sampling was performed in the morning before breakfast. Blood samples were analyzed for glucose and insulin levels using the Architect c16000 automated analyzer for clinical chemistry (Abbot). Homeostasis model assessment-insulin resistance index (HOMA_IR) was calculated as fasting insulin concentration (μU/ml) × fasting glucose concentration (mmol/L)/22.5.

3. Results and discussion

Chromato-mass-spectrometric analysis revealed 221 proteins with different IPI (international protein indices), score value going from 24 to 1700. Protein identification via UniProtKB database revealed 169 proteotypic peptides (i.e. containing amino acid sequence unique to one single protein) in the urine samples of 8 volunteers. Most of the information about the identified proteins was taken from UniProt database (http://www.uniprot.org).

In order to determine urinary proteins which are functionally important for the endothelium we analyzed our data (proteins detected in volunteers' urine and their detection rate) using ANDSystem software. This program establishes relationships between various endothelial processes and detected proteins. In this way we identified 7 endothelium-related biological processes, directly linked to 13 urine proteins (Table 1). The identifying parameters for endothelium related proteins revealed in each volunteer at two points of experiment were represented in the Supporting file.

In this paper we analyzed the relationships between the urinary proteins of interest and blood glucose, blood insulin and calculated index of insulin resistance (HOMA_IR) (Table 2). Preliminary analysis of homogeneity of variances by F-test showed for insulin a statistically significant difference in the variances between the Baseline and the End of HDBR. Therefore, correlations for insulin are not taken into consideration.

It is known that hyperglycemia induces an oxidative stress and an endothelial dysfunction with the alteration of normal endothelial antiatherogenic properties, increased extracellular matrix synthesis and