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Proteomic profiling of heat acclimation in cerebrospinal fluid of rabbit

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ABSTRACT

Heat acclimation (AC) is a phenotypic adaptation to the high ambient temperatures. So far, the physiological effects of AC have been well studied, but the molecular mechanisms underlying it, especially the proteomic studies have been rarely reported. Conducting a protein profile of cerebrospinal fluid (CSF) can facilitate the understanding of molecular pathways involved in AC and identifying stress-specific proteins as a laboratory biomarker. In this study we carried out proteomic profiling of the AC in CSF of rabbit, which would allow a deep insight into molecular signals underlying the AC. For this purpose, rabbits were subjected to AC (dry bulb temperature of (36 ± 1) °C, wet bulb temperature of (29 ± 0.5) °C, black-bulb temperature of (40 ± 1.0) °C, 100 min per day for 21 days, untreated rabbits were used as controls. We adopted a gel-free proteomic approach (iTRAQ) method to identify protein composition in CSF of rabbits with AC. In total, 1310 proteins were identified. Among which 127 were significant up-regulated and 77 were down-regulated. According to the functions, all AC-induced proteins were classified into 8 categories, including plasma protein factors, metabolism-related proteins, energy metabolism-related proteins, cell surface/intercellular matrix proteins, stress related proteins, tumor-related proteins, as well as housekeeping proteins and putative proteins. Meanwhile, a total of 21 pathways were found involved in the developing of AC. Further analysis indicated that proteins mostly close to AC were grouped into two signal pathways, the immune-related signal pathways and the carbohydrate/lipoprotein metabolism-related signal pathways. Our study was first to carry out the whole proteomic picture of AC, and screen out the critical signaling pathways involved in this physical procedure.

Biological significance: This study reported the comparative proteomic analysis of cerebrospinal fluid of rabbits between heat acclimation and normal conditions using the gel-free proteomic mass-spectrometry approach with isotope-labeled samples (iTRAQ) techniques. Mass spectrometry analysis of the proteins from heat acclimated rabbits resulted in the identification of a total of 1310 proteins, among these, 204 proteins were related to the formation of heat acclimation. These proteins were assigned to 8 categories according to their functions. Additionally, 21 pathways involved in infectious diseases, metabolism, immunology, blood circulation, transcriptional regulation and renin-angiotensin were identified by pathway analysis in heat acclimation. This study was the first to use rabbits as a model for unraveling the molecular pathways underlying the establishment of integrative heat acclimation.

1. Introduction

Heat acclimation (AC) is a phenotypic adaptation achieved via prolonged exposure to heat that improves coping and fitness during periods of severely high ambient temperatures [1]. The physiological criteria of the acclimated phenotype are decreased metabolism, heart rate (HR) and basal body temperature [2]. It is accepted that AC is a within-life, evolutionarily beneficial phenomenon, with a memory imprinted via epigenetic mechanisms [3]. However, studies of the molecular biology of AC are delayed considering its application in other research fields pertinent to human health and disease. In addition, although transcriptome studies have been widely carried out, the

Abbreviations: CSF, cerebrospinal fluid; AC, heat acclimation; MS, mass spectrometry; iTRAQ, isobaric tags for relative and absolute quantitation; MFAP4, microfibrillar-associated protein 4; HSP, heat shock protein; PASW, predictive analytics software; ANOVA, analysis of variance; SIRS, systemic inflammatory response syndrome; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TEAB, triethylammonium bicarbonate; SDS, sodium dodecyl sulfate; CVD, cardiovascular disease; LPL, lipoprotein lipase; apoC, apolipoprotein C.

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relationships between the levels of transcripts and the levels of the proteins they encode have not been examined comprehensively [4].

As the temperature-regulating center of the brain, the hypothalamus contains receptors that are sensitive to the temperature of the blood flowing through the brain. The hypothalamus contains not only the control mechanisms, but also the key temperature sensors that are considered to play important roles in heat acclimation. The idea of analyzing the critical proteins involved in heat acclimated individuals gives rise to a revealing of the core mechanism for the development of heat acclimation. Because the ependyma, cerebral pia mater and gelatinous membrane under the pia mater allow large molecules to pass through, the chemical constituents of cerebrospinal fluid (CSF) are almost the same as those in the interstitial fluid of brain tissue [5]. Therefore, component analysis of CSF is an important method for determining the cause of disease, pathologic mechanisms and effective drugs in the experimental study of the central nervous system. CSF analysis is vital because some phenomenon-related specific substances such as antibodies, specific proteins or pathogens can only be found through analyzing CSF [6].

Quantitative proteomics based on mass spectrometry (MS) is widely used in biological and clinical research for the identification of the functional modules and pathways, or the monitoring of disease biomarkers [7]. As a powerful tool for separating and identifying unprecedented numbers of proteins, MS-based proteomics can be also used for automating the identification of explicit protein isoforms in small quantities of sample. Relative quantitation of two or more samples for studies of differential protein expression is of particular importance. Quantitative results can be gained using either isobaric tags for relative and absolute quantitation (iTRAQ) or label-free methods, which are both widely used for quantitative proteomics. In this study, by analyzing the CSF from heat acclimated rabbits using an optimized iTRAQ and gel-free proteomic mass-spectrometry, followed by the previous analyses of proteins by electrophoretic techniques and candidate approaches for identification [7,8], we examined the CSF of AC rabbits for the first time to understand the molecular pathways underlying integrative AC responses during chronic exposure to new environmental and/or internal heat loads.

2. Materials and methods

2.1. Animals and experimental conditions

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). And also be approved by the Ethics Committee for Animal Experimentation of the Tianjin Institute of Health and Environmental Medicine. Sixteen male New Zealand rabbits with initial weights between 2.5 and 3.0 kg (5–6 months old) were fed laboratory chow with water ad libitum and held under light-cycled conditions (12:12 h). Rabbits were randomly assigned to the following groups. The AC group was kept in a chamber with dry bulb temperature of (36 ± 1) °C, wet bulb temperature of (29 ± 0.5) °C, black-bulb temperature of (40 ± 1.0) °C, 100 min per day for 21 days, which induces heat acclimatory homeostasis as monitored by growth rate and rectal temperatures [9]. Body weight and colonic temperature (Tc) were measured daily before and after heat exposure by using a scale dial (Xiangshan, Guangdong, China) and using a thermal probe maintained 6 cm in the rectum with a sensitivity graded (DigiSense, America) in hundredths (±0.01 °C) of Celsius degrees [10]. Meanwhile, the control group was kept in a room with temperature of 20 °C and relative humidity <60% during 21 days [10].

2.2. Drawing cerebrospinal fluid of rabbits

Immediately upon the termination of each acclimation or normothermic period, the animals were weighed and euthanized using urethane (4 ml/kg body wt via 20% urethane iv). Each animal was turned to a prone position and its head was affixed into a stereotaxic frame. The skull was exposed surgically. A dia. 2 mm, 10-cm long stainless steel needle was inserted into the aquaeductus mesencephali (P 12.5 mm, LR 0 mm, H 7 mm) and a dia. 1 mm, 10-cm long stainless steel needle was inserted into the left lateral ventricle (P 1 mm, LR 3 mm, H 7 mm) through a burr hole in the skull. The burr hole was placed at the corresponding point of the aquaeductus mesencephali and lateral ventricle according to Sawyer's graph[11]. One dia. 1.8 mm silicone catheter was introduced into the aquaeductus mesencephali, and a dia. 0.8-mm silicone catheter was introduced into the left lateral ventricle. Correct placement of the aquaeductus mesencephali catheter was indicated by the appearance of CSF in the cannula, and then 1 ml in 1 h per time of every sample CSF was withdrawn. The extraction of all the samples was performed by the same time, the CSF from different time points and each group were collected and flash freezing at −80 °C. Samples were all used for analysis within 60 days.

2.3. Protein extraction, digestion and desalting

A flowchart of the proteomics experiment comparing the protein content between CSF of rabbit AC and controls is depicted in Fig. 1A. Rabbit CSF samples were broken with sonication cracking at low temperature and then resuspended in a lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris-HCl) with 1 mM PMSF and 2 mM ethylenediaminetetraacetic acid (EDTA) (final concentration). After vortexing vigorously for 5 min, dithiothreitol (DTT) was added to a final concentration of 10 mM. After mixing, the sample was centrifuged for 20 min at 20,000 ×*g*, and supernatant was mixed well with ice-cold acetone (1:4, v/v) and 30 mM DTT. After repeating this step twice, supernatants were combined and precipitated at −20 °C overnight. The amount of protein in each CSF sample was measured using Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). CSF proteins from rabbits with the same treatment and controls were pooled to minimize individual variation and enhance signals.

Abundant CSF proteins were depleted to enhance the resolution of low expressed proteins using the ProteoMiner Protein Enrichment kit (Bio-Rad, Mississauga, Ontario, Canada) following the manufacturer's instructions. Briefly, CSF samples were centrifuged at 10,000 ×*g* for 10 min and the columns were simultaneously prepared. Clear CSF was then added to each column and rotated with an Adam nutator (Clay Adams Division of Becton Dick-Enson Co. in Parsipanny. N.J., USA) for 2 h at 20 °C to enhance protein binding to the beads; then the excessive high abundance proteins were washed away, and the medium and low abundance proteins were concentrated on their specific affinity ligands. In the end, the sample depleted of abundant proteins was eluted from the column beads into 300 μl of elution reagent. Salt and other contaminants were removed from the elute by precipitation and the enriched sample was concentrated using the ReadyPrep 2-D clean up kit (Bio-Rad, Mississauga, Ontario, Canada) according to the manufacturer's instruction. The enriched protein samples were quantified using Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

For digestion, CSF protein pellets were re-suspended in digestion buffer (100 mM triethylammonium bicarbonate, TEAB, 0.05% w/v sodium dodecyl sulfate, SDS) to a final concentration of 1 mg/ml. Total protein was measured by bicinchoninic acid assay (Sigma, St. Louis, MO). Equal aliquots (500 μg) from each lysate were then digested with trypsin overnight at 37 °C (Sigma; 1:40 w/w added at 0 and 2 h) and lyophilized.

2.4. iTRAQ labeling

The iTRAQ labeling of peptide samples derived from rabbits of AC and controls were performed using iTRAQ Reagent 4-plex kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Six samples (three biological replicates for rabbits of AC and controls,