Journal of Pharmaceutical and Biomedical Analysis 95 (2014) 68–75

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Characterization of rational biomarkers accompanying fever in yeast-induced pyrexia rats using urine metabolic footprint analysis

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ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form 13 February 2014
Accepted 15 February 2014
Available online 26 February 2014

Keywords:
Metabolic footprint analysis
Biomarkers
Yeast-induced pyrexia
Ultra performance liquid chromatography
Quadrupole time-of-flight mass spectrometry

ABSTRACT

Fever is a prominent feature of diseases and is an ongoing process that is always accompanied by metabolic changes in the body system. Despite the success of temperature regulation theory, the underlying biological process remains unclear. To truly understand the nature of the febrile response, it is crucial to confirm the biomarkers during the entire biological process. In the current study, a 73-h metabolic footprint analysis of the urine from yeast-induced pyrexia rats was performed using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Potential biomarkers were selected using orthogonal partial least squares-discriminate analysis (OPLS-DA), the rational biomarkers were verified by Pearson correlation analysis, and the predictive power was evaluated using receiver operator characteristic (ROC) curves. A metabolic network constructed using traditional Chinese medicine (TCM) grammar systems was used to validate the rationality of the verified biomarkers. Finally, five biomarkers, including indoleacrylic acid, 3-methyluridine, tryptophan, nicotinuric acid and PI (37:3), were confirmed as rational biomarkers because their correlation coefficients were all greater than 0.87 and because all of the correlation coefficients between any pair of these biomarkers were higher than 0.75. The areas under the ROC curves were all greater than 0.84, and their combined predictive power was considered reliable because the greatest area under the ROC curve was 0.968. A metabolic network also demonstrated the rationality of these five biomarkers. Therefore, these five metabolites can be adopted as rational biomarkers to reflect the process of the febrile response in inflammation-induced pyrexia.

1. Introduction

Fever is one of the main acute-phase reaction symptoms in a variety of diseases, particularly in infectious diseases, inflammatory diseases and in autoimmune diseases [1,2]. Although there are two thermoregulatory circuitries that have been reported, *i.e.*, the humoral pathway and the neural pathway [3], numerous endogenous substances participating in the febrile response and their dynamic changing process have not been clarified. Screening rational biomarkers from numerous metabolites that are directly correlated with body temperature and exploring the network relation between biomarkers and febrile response-related inflammatory mediators and/or cytokines that play determinant roles is important for understanding the mechanism of pyrexia.

At present, several pyrexia-related animal models have been established and validated to explore the pathogenesis and pathophysiology of pyrexia [4–6]. Among these models, yeast-induced pyrexia has been widely utilized to study the development of the pyretic bodies induced by inflammation [7–9]. Yeast-induced

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http://dx.doi.org/10.1016/j.jpba.2014.02.011
0731-7085/

pyrexia has been reported as a pathogenic fever that leads to an intense inflammatory reaction caused by ulceration at the injection site [10]. Until now, the mechanism of the yeast-induced pyrexia model has not been completely clarified, in spite of its wide adoption.

In our previous study, we have proven that the yeast-induced febrile response is a dynamic pathological developing process, during which the changing trend of metabolic profile clustering was consistent with the changing trend of the rectal temperature in yeast-induced pyrexia rats, and impaired tryptophan metabolism was demonstrated to be related to the fever [11]. With subsequent research, we found that the selection of time-points was important for utilizing metabolomic tools to reveal the underlying biological processes of acute metabolic diseases, such as fever [12]. If only one single time-point instead of for the continuous changing process was adopted, a cross-sectional for the study object would occur. Therefore, based on our previous study, we perform a dynamic study to reveal for the first time the dynamic changing process of organisms.

In metabolomic research, the course from potential biomarkers to clinical indicators or terminal indexes is complex and time-consuming. Metabolites identified from an early stage of metabolomics should undergo larger, prospective, externally validations in clinical cohorts before their future employment as practical biomarkers [13], thus demanding numerous studies [14]. Among the many published metabolomic studies that focus on biomarker discovery, few researchers have reported to the process by which the selected range of potential biomarkers is narrowed. Due to the complexity of the fever mechanism, it is difficult to elucidate the exact metabolism of potential biomarkers. Fortunately, the available Pearson correlation analysis, receiver operator characteristic (ROC) curves and traditional Chinese medicine (TCM) grammar systems provide good selecting and verifying features. Pearson correlation analysis can be used to assess the relation between potential biomarkers and clinical indicators. ROC curve analysis is widely considered to be the most objective and statistically valid method for biomarker performance evaluation [15]. Moreover, TCM grammar systems, which provide formal tools to study the entangled hierarchies in biological systems and to control the generation of emergence in certain conditions by entity grammar systems [16], can integrate the concerned pieces of parsed knowledge to better elucidate the relations between biomarkers and pyrexia. In this paper, we investigated for the first time the relations between potential biomarkers at different time-points using a metabolic footprint and a pharmacological index, aiming to discover more rational biomarkers of the febrile response in yeast-induced pyrexia rats. In addition, the filtered biomarkers were validated using ROC curve analysis and a network constructed using TCM grammar systems. This network was composed of proven inflammatory mediators and cytokines relating to the febrile response. The results obtained in current study are expected to provide a practical strategy to identify biomarkers for studying the mechanism of inflammation-induced pyrexia and other acute metabolic diseases.

2. Materials and methods

2.1. Chemicals and reagents

Yeast was purchased from Mauri Food Co. Ltd. (Hebei, China). HPLC grade methanol and acetonitrile were acquired from Baker Company (Baker Inc., USA). Ultra high purity water was prepared by Millipore-Q SAS 67120MOLSHEIM (France). HPLC grade formic acid (No. 7000027413) was obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

2.2. Study protocol and sample collection

All protocols and the care of the rats were in accordance with the institutional guidelines for animal use in research. Male Sprague-Dawley rats (200 ± 20 g) were obtained from Beijing Weitonglihua Laboratory Animal Technology Co. Ltd. (Beijing, China). All of the rats were raised at a standard temperature ($23 \pm 2\,^{\circ}C$) and humidity ($60 \pm 5\%$) in a controlled room with a light/dark cycle of 12 h. The rats underwent an adaptation period of seven days, during which the rats were fed commercial feed and drank water *ad libitum*. Then, the rats were transferred to individual metabolic cages and allowed to acclimatize for three additional days. During this period, the rats' rectal temperatures were measured three times per day using a digital thermometer (Shanghai Huachen Medical Instruments Co. Ltd.) for monitoring the regular rhythm of body temperatures. The rats with temperature differences that were greater than $0.5\,^{\circ}C$ were excluded. Sixteen qualified rats were chosen and randomly divided into two groups, including the normal control group (CG) and the pyrexia model group (PG). The PG rats were subcutaneously injected with a 20% aqueous suspension of yeast (15 mL/kg) in the back of the rats. The CG rats were given an equal volume of 0.9% saline in parallel. The rectal temperature was measured, and the urine was collected at the time-points of 0, 5, 9, 13, 25, 37, 49, 61 and 73 h after administration. Each urine sample was centrifuged at 14,000 rpm for 10 min at $4\,^{\circ}C$, and the supernatant was collected and stored at $-20\,^{\circ}C$ until use.

2.3. Urine sample preparation and UPLC Q-TOF/MS analysis

Before the analysis, urine samples were thawed at room temperature, diluted at a ratio of 2:1 (v/v) using ultra high purity water, vortex mixed and centrifuged at 14,000 rpm for 10 min at $4\,^{\circ}C$. Then, the supernatants were subjected to the UPLC Q-TOF/MS system for analysis. The analysis was performed using a Waters Acquity^{TM} Ultra Performance LC system (Waters Corp., Milford, MA) connected to a Xevo^{TM} G2-Q-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) that was equipped with an electrospray ionization (ESI) source, which was operated in the positive mode. The separation conditions and mass spectrometry parameters were described in our previous reports [11,17]. An MS^{E} technology was used for the data collection, and the parameters were set as follows: function 1, 6 eV collision energy; function 2, collision energy ramp of 20–40 eV.

2.4. Data analysis

The raw mass data were analyzed using the Applied Waters Markerlynx XS software (Waters Corporation, Milford, MA, USA), which could transform the raw data into a single matrix containing aligned peaks with t_r-m/z pairs, normalized peak intensities and sample names. For extracting data from the raw file and for detecting potential markers, the retention time range was set at 0–11.5 min; the mass range was set at 50–1000 amu; and the mass tolerance was set at 0.01. For detecting chromatographic peaks in the Apex Track Peak, the peak width at 5% height was set at 1.00, and the peak-to-peak baseline noise was set at 0.00. For collecting parameters, the marker intensity threshold was set at 1000 cps; the mass window was set at 0.02 amu; and the retention time window was 0.20 min. The noise elimination level was 6. This process provides an alignment of drifts (retention time and accurate mass) in data and ensures that a chromatographic peak is identified using identical parameters for each sample. Subsequently, a list of intensities or peak areas of the peaks was then generated for the first chromatogram, using the t_r-m/z pairs as identifiers. This procedure was applied for each UPLC/MS analysis. The ion intensities or peak area for each peak detected were also normalized within each