Epilepsy Research 126 (2016) 83–89

Contents lists available at www.sciencedirect.com

Epilepsy Research

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

GC–MS–Based metabolomics discovers a shared serum metabolic characteristic among three types of epileptic seizures

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 2 July 2016
Accepted 14 July 2016
Available online 16 July 2016

Keywords:
Seizure
Metabolic profile
Lactate
Glyceraldehyde
Trans-13-octadecenoic acid

ABSTRACT

Objective: Understanding the overall and common metabolic changes of seizures can provide novel clues for their control and prevention. Here, we aim to investigate the global metabolic feature of serum for three types of seizures.
Methods: We recruited 27 patients who had experienced a seizure within 48 h (including 11 who had a generalized seizure, nine who had a generalized seizure secondary to partial seizure and seven who had a partial seizure) and 23 healthy controls. We analyzed the global metabolic changes of serum after seizures using gas chromatography-mass spectrometry-based metabolomics. Based on differential metabolites, the metabolic pathways and their potential to diagnose seizures were analyzed, and metabolic differences among three types of seizures were compared.
Results: The metabolic profiles of serum were distinctive between the seizure group and the controls but were not different among the three types of seizures. Compared to the controls, patients with seizures had higher levels of lactate, butanoic acid, proline and glutamate and lower levels of palmitic acid, linoleic acid, elaidic acid, *trans*-13-octadecenoic acid, stearic acid, citrate, cysteine, glutamine, asparagine, and glyceraldehyde in the serum. Furthermore, these differential metabolites had common change trends among the three types of seizures. Related pathophysiological processes reflected by these metabolites are energy deficit, inflammation, nervous excitation and neurotoxicity. Importantly, transamination inhibition is suspected to occur in seizures. Lactate, glyceraldehyde and *trans*-13-octadecenoic acid in serum jointly enabled a precision of 92.9% for diagnosing seizures.
Conclusions: There is a common metabolic feature in three types of seizures. Lactate, glyceraldehyde and *trans*-13-octadecenoic acid levels jointly enable high-precision seizure diagnosis.

1. Introduction

As defined by the International League against Epilepsy (ILAE), epilepsy is defined as "a group of neurological disorders characterized by an enduring predisposition to generate epileptic seizures" (Fisher et al., 2014). Epilepsy, with high morbidity and a long duration, seriously threatens people's health and quality of life. According to the WHO, there are approximately 50 million epilepsy patients worldwide (Brodie et al., 1997), and more than 30% of all epilepsy patients cannot control their seizures with current medications (Kwan and Brodie, 2000). The key factors that provoke seizures are not yet clear, making seizures a poorly controlled and unpredictable condition.

Metabolism is among the first areas affected after a seizure, which can then lead to different deleterious consequences. What is the global metabolic feature of seizures? Are there any common metabolic features of various seizures? Answering these questions increases the possibility of improving the knowledge of the pathophysiology of seizures and offers some novel clues for controlling and preventing different types of seizures.

High-throughput data-based metabolomics, a technology for studying global changes of endogenous metabolites under pathological or environmental stimulation, has been increasingly used to screen for biomarkers and key factors of the genesis and development, of many diseases (Nicholson et al., 1999). Metabolomics has also been applied to investigate changes in the metabolism of epilepsy patients and the effects of anti-seizure drugs (Al Zweiri et al., 2010; Mimrod et al., 2005; Wei et al., 2012). As yet, there

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http://dx.doi.org/10.1016/j.eplepsyres.2016.07.003
0920-1211/

have been no studies concerning metabolome changes in the serum post-seizures. Serum is commonly considered to be a pool of metabolites that reflect systematic deregulation in patients. The bi-directional flow of endogenous metabolites between blood and brain compartments makes it possible for the abnormalities in metabolite serum levels to reflect those in the brain.

As epilepsy is a paroxysmal diseases, its seizures may have a common metabolic change after they attack. Therefore, in this study, we aimed to investigate the global, common metabolic profile of serum in different types of seizures. First, we recruited 27 patients who experienced a seizure within 48 h (including 11 patients with a generalized seizure, nine patients with a generalized seizure secondary to a partial seizure and seven patients with a partial seizure only) and 23 age- and sex-matched healthy controls. Second, blood was collected from these subjects and the metabolic profiles of serum were analyzed using gas chromatography-mass spectrometry (GC–MS)-based metabolomics. Third, due to differentially expressed metabolites between all patients and the controls, the metabolic pathways and their potential to diagnose seizures were analyzed. Finally, the metabolic profiles and differential metabolites were further compared between each type of seizure with the controls and among three types of seizures to find a shared metabolic characteristic.

2. Patients and methods

2.1. Subjects

This study was approved by the ethics committee of the 1st Affiliated Hospital of Shantou University Medical College, and written informed consent was obtained from all subjects. Twenty-seven patients who had a seizure within 48 h and 23 age- and-sex-matched healthy controls were enrolled from the 1st Affiliated Hospital of Shantou University Medical College. Epilepsy was diagnosed according to a practical clinical definition of epilepsy released by the International League Against Epilepsy (ILAE) in 2014 (Fisher et al., 2014). Seizures were confirmed by clinical manifestations, which included recurrent instantaneous loss of consciousness, alteration of perception or impairment of psychic function, convulsive movements and disturbance of sensation, as well as epileptoid discharge in the electroencephalogram (EEG). The NHS score was calculated according to the National Hospital Seizure Severity Scale (NHS) to evaluate the severity of epilepsy (O'Donoghue et al., 1996). The seizure type was classified according to the report of the ILAE Commission on Classification and Terminology, 2005–2009 (Berg et al., 2010).

2.2. Sample collections

Blood samples of all subjects were obtained within 48 h after seizures by venipuncture after fasting overnight and collected in glass tubes containing coagulant. After 10 min at room temperature, the serum was separated by centrifugation at 3000 rpm for 5 min at 4 °C and stored at minus 80 °C.

2.3. Serum preparation

The serum was extracted with methanol as previously described (Jiye et al., 2005). Briefly, 100 μL of serum was mixed with 900 μL of a solvent mixture (methanol: H_2O: 8:1, v/v), vortexed for 15 s and centrifuged at 12,000*g* for 10 min at 4 °C. Then, 200 μL of the supernatant was transferred to a GC vial, 20 μL of internal standard D-norleucine (0.5 μM mL^{-1}) was added, and the sample was mixed well and dried at 70 °C under a mild flow of pure nitrogen. After adding 20 μL of methylene chloride, the samples were blown dry for a few additional minutes to ensure absolute dryness. Next, the aliquot was oximated by adding 30 μL of methoxamine hydrochloride (15 mg mL^{-1}) in pyridine and incubating for 16 h at room temperature. Later, 30 μL of N-methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (MTBSFA) with 1% trimethylchlorosilane (TMCS) was added, and the sample was vortexed for 30 s and kept at 37 °C for 1 h for trimethylsilylation. A 1-μL aliquot of the derivatized solution was used for GC–MS analysis.

2.4. Metabolic profiling by GC–MS

The metabolic profiling analysis was conducted on a GC–MS system consisting of an Agilent 6890N GC system, connected to an Agilent 5975c single quadrupole MSD. A DB-5MS capillary column (Agilent, USA) was used for the separation of metabolites. One microliter of derivatized sample was injected in splitless mode. High-purity helium was used as the carrier gas at a constant flow rate of 60 mL h^{-1}. The GC oven temperature was programmed with an initial temperature at 60 °C for 2 min, which was then increased to 285 °C at 5 °C min^{-1} and maintained for 2 min at 285 °C. The temperature of the injection port, the transfer interface and the EI source temperature were set to 230, 290 and 230 °C, respectively. The selected mass range was set to 50–600 *m*/*z* with electron impact ionization (70 eV), and the selected scan speed was 0.99 scans per second.

Each acquired chromatogram was analyzed using ChemStation software (Agilent). Metabolites were identified directly by comparing the similarities and differences between the theoretical mass spectra and experimental mass spectra in the 2.0 NIST (National Institute of Standards and Technology) library. The peak area of the chromatogram in every sample was normalized using the internal standard (D-norleucine) peak area.

Before detection, precision and stability were evaluated to ensure the quality of analysis. The precision of the GC–MS system was investigated by five successive injections of one derivatized sample on the same day (intra-day). The stability of deposition and treatment (inter-day) was determined in five replicates of the same serum sample on five successive days. Both results were expressed by the relative standard deviation (RSD) of the peak area of principal metabolites. The intra-day RSDs of six main metabolites, namely, phosphate, proline, lactate, alanine, glutamate and hexadecanoic acid were 3.11%, 2.20%, 3.43%, 0.35%, 3.16% and 3.63%, respectively. The inter-day RSDs of these metabolites were 2.33%, 6.41%, 5.34%, 3.54%, 4.89%, and 12.31%, respectively.

2.5. Data processing

2.5.1. Multivariate statistical analysis

GC–MS raw data was processed by the freely available software package XCMS in R language. An Analytical Instrument Association (AIA) format file was converted from the chromatogram in each test with ChemStation software. A txt format file was established and the data were then imported into the SIMCA-P program (version 13.0 Umetrics) for multivariate analysis. Partial least squares discriminant analysis (PLS-DA) was applied with unit variance (UV) scaling. The parameters of the models, such as the R^2X, Q^2Y and the R^2Y and Q^2Y intercepts, were investigated to ensure the quality of the multivariate models and to avoid the risk of over-fitting.

2.5.2. Univariate statistical analysis

A *t*-test was performed to compare the relative amount (peak area) of identified metabolites between the patients and the controls, and between each type of seizures and the controls.