Process Biochemistry 58 (2017) 78–84

Contents lists available at ScienceDirect

Process Biochemistry

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

A mass spectrometry-based strategy combined with bioinformatics: A simple preclinical model for profiling valproic-acid-induced major proteins and modifications in human liver cells

Chun-Yi Wu[a], Tusty-Jiuan Hsieh[b], Wei-Lung Tseng[c], Chun-Lan Keng[d], Chi-Yu Lu[a,d,e,f,*]

[a] Department of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[b] Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[c] Department of Chemistry, College of Science, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[d] Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[e] Institute of Medical Science and Technology, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[f] Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan

ARTICLE INFO

Keywords:
Valproic acid
Hepatotoxicity
Reactive oxygen species
Indicator

ABSTRACT

The clinical use of valproic acid (VA) as an antiepileptic drug is associated with adverse effects such as hepatotoxicity and encephalopathy. These potential complications may be caused by reactive oxygen species (ROS) produced during the metabolic processing of VA. In this study, we used screening to identify cellular protein markers of VA-induced oxidative stress in human liver cells. To confirm that the protein modifications were induced by VA, nano-ultra-performance liquid chromatography (nanoUPLC) coupled with tandem mass spectrometry (MS/MS) was used for structural identification. Protein modifications induced by oxidative stress were found to involve crotonaldehyde, 4-hydroxynonenal, an oxygen atom, nitric oxide, and the nitro and acetyl groups. The data on the above-mentioned protein modifications may serve as indicators of apoptosis, oxidative stress, or other adverse reactions induced by the clinical use of VA.

1. Introduction

Valproic acid (VA) is an effective anticonvulsant and antiepileptic drug that is particularly useful for managing generalized forms of epilepsy [1]. Nonetheless, long-term use of VA is associated with adverse reactions such as hepatotoxicity, fatal hemorrhagic pancreatitis, bone marrow suppression, hyperammonemia encephalopathy, hepatitis, agranulocytosis, thrombocytopenia, polycystic ovary disease, menstrual aberrations, hyperandrogenism, and peripheral insulin resistance [2,3]. Biotransformation of VA proceeds in two main phases: the oxidative reaction phase (phase I, including oxidation, reduction, and hydrolysis) and the conjugative reaction phase (phase II, including glucuronidation and conjugation with glutathione, carnitine, coenzyme A, and/or amino acids such as glycine or glutamic acid) [4]. Many studies have shown that oxidative stress induced by VA can cause apoptosis [5–9]. Treatment with VA is a popular example of drug-induced liver injury by mitochondrial dysfunction; the mitochondrial alterations can induce mild-to-fulminant hepatic cytolysis and steatosis [10–13]. To prevent VA-induced hepatotoxic processes and other severe side effects, the use of VA for clinical treatment of a disease must be monitored carefully.

Proteins are among the most abundant components of living cells and are potential indicators of reactive oxygen species (ROS). Because ROS produced by VA can cause apoptosis [5–9], the protein modifications induced by VA must be elucidated for an improved understanding of the processes of oxidative stress or apoptosis. In this work, nano-ultra-performance liquid chromatography (nanoUPLC) coupled with tandem mass spectrometry (MS/MS) was used to identify protein modifications. The protein modifications identified by nanoUPLC–MS/MS were then evaluated for use as cellular markers of drug-induced oxidative stress.

2. Experiment

2.1. Materials and chemicals

VA (2-propyl pentanoic acid), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium bicarbonate (NH_4HCO_3), acetone, acetonitrile, and formic acid (FA) were purchased from Merck (Darmstadt, Germany). All

* Corresponding author at: 100, Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan.
E-mail address: cylu@kmu.edu.tw (C.-Y. Lu).

http://dx.doi.org/10.1016/j.procbio.2017.04.021
Received 6 November 2016; Received in revised form 9 April 2017; Accepted 10 April 2017
Available online 20 April 2017
1359-5113/

reagents were of analytical grade. Sequence grade trypsin was acquired from Promega (Madison, WI, USA). Deionized water for preparation of reagents was produced by a Millipore Milli-Q water purification system (Bedford, MA, USA).

2.2. Preparation of working solutions

FA (0.1%) and NH_4HCO_3 (25 mM) solutions were prepared in water. DTT (25 mM) and IAA (25 mM) were prepared in a 25 mM NH_4HCO_3 aqueous solution. Sequence grade trypsin (0.1 μg/μL) was prepared in the buffer supplied by the manufacturer.

2.3. Equipment

For protein analysis, the peptide sequences were identified by nanoUPLC connected to an LTQ Orbitrap Discovery hybrid Fourier Transform Mass Spectrometer (Thermo Fisher Scientific, Inc., Bremen, Germany). The nanoUPLC system was purchased from Waters (Milford, MA, USA) and was operated with a trapping column (Symmetry C18, 5 μm, 180 μm × 20 mm) and an analytical column (BEH C18, 1.7 μm, 75 μm × 150 mm). The LTQ Orbitrap was operated in positive ion mode with a nanospray source. The spray capillary temperature was set to 200 °C at resolution 30000. Voltage at the source, tube lens, and capillary was set to 2.3 kV, 80 V, and 28 V, respectively.

2.4. Cultured cells treated with VA

To study VA-induced cytotoxicity, cells in culture were treated with VA at 35 μg/mL (prepared in DMSO) [14]. Briefly, Huh-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with normal glucose (5.5 mM), 10% fetal bovine serum, and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) in an incubator at 37 °C and 5% CO_2. The number of cells grown in each 10-cm tissue culture dish reached ~6 million at 60% confluence. Huh-7 cells were treated for 72 h with VA at 35 μg/mL in the experimental group and were treated with DMSO in the control group. After treatment, the cells were washed with phosphate-buffered saline and stored at −80 °C until analysis.

2.5. Preparation of a sample for identifying proteins and protein modifications

The protein identification procedures were conducted as follows. First, Huh-7 cells were lysed with RIPA lysis buffer (50 μL, Millipore, CA, USA) for 30 min. The cell lysate was then transferred to a fresh tube and centrifuged at 10,000*g* for 10 min. The supernatant (10 μL) was mixed with acetone (90 μL), and the tube was centrifuged at 10,000*g* for another 10 min. Protein pellets were dried and redissolved in 25 mM NH_4HCO_3. For protein reduction and alkylation, a solution containing ~5 μg of protein (16 μL) was incubated with DTT (2 μL) at 25 °C for 30 min. After addition of an IAA solution (2 μL), the mixture was incubated at 25 °C for another 30 min. Finally, a freshly prepared trypsin solution (2.5 μL) was added, and the resulting protein solution was incubated at 37 °C for 16 h. For protein identification, the peptide solution (2 μL) was injected into the nanoUPLC–MS/MS system.

2.6. Protein identification by nanoUPLC–MS/MS

For protein identification, the nanoUPLC conditions were as follows. After desalting of peptide mixtures with an on-line trapped column, we performed peptide separation on a nano-flow reverse-phase C18 column. The desalting procedure was performed for 3 min at a flow rate of 5 μL/min with 0.1% FA. Tryptic peptides were then separated on an analytical column at a flow rate of 300 nL/min. Mobile phase A was 0.1% FA, and mobile phase B was 100% acetonitrile (containing 0.1% FA). The gradient conditions were the following: t = 0 to 2 min, hold B at 1%; t = 2 to 40 min, increase B from 1% to 45%; t = 40 to 60 min, increase B from 45% to 85%; t = 60 to 70 min, hold B at 85%; t = 70 to 90 min, decrease B from 85% to 1%; t = 90 to 120 min, hold B at 1%. The LTQ Orbitrap conditions were as follows. The full scan mass range was set to *m/z* 400–2000 in profile mode at resolution 30,000. To acquire the MS/MS spectra, precursor ions in the linear ion trap were fragmented with helium (collision energy 35 eV) as the collision gas, with the lock mass ion signal was set to *m/z* 445.12. For collision-induced dissociation, four of the most abundant multiple-charge ions were selected. Raw data files were processed in the Mascot Distiller software (Matrix Science Inc, Boston, MA, USA) to create the peak lists, which were then uploaded to the Mascot server (Matrix Science Inc.) for protein identification.

2.7. SDS-PAGE and Western blot analysis

To analyze the proteins, cells were lysed in RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). Protein concentration was measured using the BCA Protein assay kit (Thermo Fisher Scientific, Inc.). For analysis of each specific protein, 30 μg of cell lysate was loaded and separated on 15% SDS-polyacrylamide gels. After transference to the PVDF membranes, the proteins of interest were detected using the corresponding antibodies. Images of protein expressions on the blots were quantified by ImageJ software (Rasband, 1997). Cytochrome c and GST omega 1 antibodies were purchased from Abcam (Cambridge, MA, USA).

3. Results and discussion

One cause of the unusual hepatotoxic effects and other adverse effects of VA is oxidative stress induced by the biotransformation of VA in the liver. Under oxidative stress, ROS modify major proteins in living cells. In this study, nanoUPLC–MS/MS was coupled with a database search to determine whether VA-induced protein modifications and profiles in human liver cells can be used as cellular markers of drug-induced oxidative stress. For identification of such proteins and protein modifications, cell samples are analyzed in triplicate by LC–MS/MS. Fig. 1 is a schematic diagram of the proposed procedure for the use of nanoUPLC–MS/MS to identify protein profiles and modifications induced by VA.

3.1. Identification of protein profiles induced by VA

Because the production of VA and its metabolites is related to oxidative stress or to apoptosis [5–9], nanoUPLC–MS/MS was used to profile proteins in Huh-7 cells treated with VA. Protein data obtained by means of a database search of the proteins identified by nanoUPLC–MS/MS were imported into the Ingenuity Pathway Analysis Tool (IPA Tool; Ingenuity Systems, Redwood City, CA, USA; http://www.ingenuity.com). Fig. 2A is a Venn diagram comparing the distributions of two protein groups differentially expressed between Huh-7 hepatocytes incubated with and without VA. The comparison shows that 173 proteins were common for the two protein groups; of these, 150 were differentially expressed only in VA treated samples whereas 142 proteins were differentially expressed only in control samples. Fig. 2B shows a comparison of protein expression profiles between VA-treated samples and control samples. The most abundant proteins were cytoplasmic and nuclear proteins.

The subcellular locations of proteins in VA-treated cells were identified and summarized with the UniProt ID mapping tool (http://www.uniprot.org/). Fig. 3A is a classification diagram showing that protein expression was the strongest in the cytoplasm and in the nucleus. The biological processes were classified by means of the STRAP software (http://cpctools.sourceforge.net) to search for annotations of the proteins. Fig. 3B shows the distribution of the Gene