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UPLC-MS-based serum metabonomics for identifying acute liver injury biomarkers in Chinese miniature pigs

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HIGHLIGHTS

- The acute liver injury experimental pig model is similar to humans.
- Four groups of biomarkers were identified: conjugated bile acids, LPCs, PCs and FAAs.
- A UPLC-Q/TOF-MS based metabonomics approach was used.
- Bile acid metabolic pathways, lipid metabolism, and FAAs metabolism disorder were the major metabolic effects.
- The unsupervised PCA and the supervised OPLS analyses were used.

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ABSTRACT

Metabonomics has emerged as an important technology for exploring the underlying mechanisms of diseases and screening for biomarkers. In this investigation, to comprehensively assess metabolite changes in D-galactosamine (GalN)-induced liver injury in Chinese miniature pigs and to increase our understanding of physiological changes in normal and pathological states, we used ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) to analyze metabolites and identify biomarkers in serum. Blood samples were collected both from 18 h after GalN treatment pig and from control group pig. We performed multivariate analyses on the metabolite profiles to identify potential biomarkers of acute liver injury, which were then confirmed by tandem MS. Based on "variable of importance in the project" (VIP) values and S-plots, four groups of biomarkers were identified – namely conjugated bile acids, lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs) and fatty acid amides (FAAs) – that were present at significantly different levels in the control and GalN-induced groups. LPCs, PCs, and FAAs showed marked decreases in the GalN-treated group, whereas conjugated bile acids in the treated group showed considerable increases. Taken together, our results suggested that obvious metabolic disturbances occur during acute liver injury, which provided novel insights into the molecular mechanism(s) of D-galactosamine (GalN)-induced liver injury, and will facilitate future research and management of liver injury.

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; BPI, base peak intensity; CDP-choline, cytidine diphosphate choline; CHO, cholesterol; Cr, creatinine; ESI, electrospray ionization; FAAH, fatty acid amide hydrolase; FAAs, fatty acid amides; FIB, fibrinogen; GalND-, galactosamine; GCA, glycocholic acid; GC–MS, gas chromatography–mass spectrometry; GCDCA, glycochenodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; LPCs, lysophosphatidylcholines; MS, mass spectrometry; NMR, nuclear magnetic resonance; OPLS, orthogonal partial least squares; PCs, phosphatidylcholines; PCA, principal components analysis; PEMT, phosphatidylethanolamine-N-methyltransferase; PLA2, phospholipase A2; PT, prothrombin time; QC, quality control; Q-TOF, quadrupole-time of-flight; SST, serum separator tubes; TBA, total bile acids; TBil, total bilirubin; TUDCA, tauroursodeoxycholic acid; UPLC, ultraperformance liquid chromatography; VIP, variable of importance in the project.

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1. Introduction

The liver is a vital organ that is present in mammals and many other vertebrates. It carries out many critical functions, including the manufacture of essential proteins, detoxification, decomposition of red blood cells, and the production of biochemical compounds, many of which are necessary for normal vital functions. The liver is the hub of systemic metabolism and is necessary for survival. Upon injury to the liver not only its function but also other systemic metabolic pathways are affected. Liver disease is a major threat to human health (Smets et al., 2008). Liver failure is an extreme manifestation of severe liver injury, and a rare liver disorder with an 80% mortality rate. Generally, liver injury and functional assessment are based on clinical symptoms, serum biomarkers, and, occasionally, invasive liver biopsies. "Traditional" serum clinical biomarkers, such as alanine aminotransferase (ALT), are commonly used to evaluate liver injury. ALT is abundant in the cytosol of hepatocytes; as a biomarker, it is generally sensitive but not necessarily specific for liver injury (Ozer et al., 2008). Furthermore, traditional serum clinical biomarker levels may not always correlate well with the degree of injury. Thus, there is a continuing need for new biomarkers of liver injury and function.

The "-omics" technologies, such as genomics, proteomics, and metabonomics, have been used successfully in animal studies to develop biomarkers of liver injury (Bando et al., 2011; Fella et al., 2005; Shi et al., 2010; Want et al., 2010; Yang et al., 2008). Metabonomics is an important component of systems biology and provides quantitative measures of global changes in the metabolic profiles of individuals in response to pathophysiological stimuli or genetic modification (Brindle et al., 2002; Ganti et al., 2012; Nicholson et al., 1999; Sreekumar et al., 2009). It complements genomics, transcriptomics, and proteomics. Metabonomics measures the "omics cascade" downstream products to provide information that is not accessible through the other "omics." Metabonomics facilitates investigation of human biology and health by analyzing body fluids and tissues and can identify specific biomarkers for the early detection, diagnosis, and prediction of disease prognosis (Cortes et al., 2010; Garcia-Canaveras et al., 2011). Metabonomics is a rapidly evolving method of determining changes in the concentrations of some small molecules (molecular weight < 1000 Da) in biofluids (such as serum, urine, plasma), tissues, and cells (Griffin and Nicholls, 2006). Because of these advantages, metabonomics is a sensitive and powerful tool for biomarker discovery and for understanding the pathophysiology of disease (Li et al., 2008; Yang et al., 2008).

D-galactosamine (GalN)-induced acute liver injury is a well-characterized experimental model, which produces hepatic failure similar to human viral hepatitis. The underlying mechanism involves an inadequate supply of the nucleotide uridine, which inhibits protein and nucleic acid synthesis (Coen et al., 2007). As large mammals, pigs are similar to humans in terms of anatomical structure, physiology, and clinical manifestations. The pig model of acute liver injury induced by GalN was described in our previous work (Cao et al., 2012). The purpose of this study was to investigate GalN-induced acute liver injury in Chinese miniature pigs using a metabonomics approach based on ultra-performance liquid chromatography–mass spectrometry (UPLC-MS), and to identify biomarkers of acute liver injury.

2. Materials and methods

2.1. Reagents and materials

GalN was purchased from Hanhong Chemical (Shanghai, China). Chromatography-grade acetonitrile, formic acid, and leucine-enkephalin were purchased from Sigma–Aldrich (St. Louis, MO). Distilled water was purified using a Milli-Q ultrapure water machine (Millipore, Bedford, MA). All standard samples were purchased from Sigma–Aldrich or originated from previous studies in our laboratory.

2.2. Animals

Animal procedures were approved by the Animal Care Ethics Committee of the First Affiliated Hospital, Zhejiang University. All animals received humane care according to the criteria of the "Guide for the Care and Use of Laboratory Animals."

Twelve male Chinese experimental miniature pigs (10–12 kg) were used; they were purchased from Beijing Agriculture University (Beijing, China). The pigs were housed in singular standard cages in a controlled environment with an air-conditioned room temperature of 22 ± 2 °C and a relative humidity of 65 ± 5% under a 12/12-h light/dark cycle. The animals were acclimatized for 1 week before the experiments. Standard laboratory chow and water were provided ad libitum.

2.3. GalN-induced acute liver injury model

An acute liver injury model was induced in the pigs by administration of GalN via the jugular vein as described previously (Cao et al., 2012). Briefly, the 12 pigs were randomly divided into two groups and fasted for 12 h before treatment. The treatment group ($n = 6$) was induced by administration of GalN at a dose of 1.5 g/kg body weight via jugular vein catheterization, whereas the control group ($n = 6$) underwent a sham administration procedure with an equal volume of normal saline and no GalN. After induction, the pigs were returned to their cages. During the whole course of the experiment, the pigs' general condition, such as movement, food intake, and mental condition, was observed closely. Survival time was calculated from the time of administration of GalN.

2.4. Sample collection and preparation

Blood samples were collected from the control group and GalN treatment group at 18 h after the administration of GalN in Vacutainer SST tubes, BD Vacutainer blood collection tubes, and Vacutainer coagulation tubes (Becton, Dickinson and Company, Franklin Lakes, USA). When blood samples were collected, they were stored at 4 °C immediately and transported to the central clinical laboratory. The samples for serum were left on the laboratory bench for 30 min before being centrifuged (3000 × g, 10 min, 4 °C). Serum samples (500 μL), blood collection tubes, and coagulation tubes were sent to the central clinical laboratory of the First Affiliated Hospital for biochemical assays. The remaining serum was transferred to fresh microcentrifuge tubes and stored at −80 °C until metabonomics analysis.

Prior to metabonomics analysis, all serum samples were thawed at 4 °C and the samples (200 μL) were then mixed with 600-μL ice-cold acetonitrile, mixed vigorously, and centrifuged (14,000 × g, 10 min, 4 °C). Finally, the supernatants were transferred to glass tubes and stored at 4 °C for UPLC-MS analysis. Quality control (QC) samples were pooled from 30 μL of supernatants drawn from each sample. The QC sample was tested five times before the analysis to condition the column and was inserted every four samples throughout the run. The results of the five consecutive runs of the QC samples are shown in Fig. 1; the QC chromatograms overlapped tightly, retention times were stable, with no obvious drift, and most of the peaks were sharp and symmetrical, indicating that the metabolic analysis system was highly reproducible.

2.5. UPLC-QTOF-MS analysis of serum

Chromatographic separations were performed on a Waters (Milford, MA) Acquity ultra-performance liquid chromatography system equipped with an Acquity UPLC BEH C18 analytical column (i.d. 2.1 mm × 100 mm, particle size 1.7 μm, pore size 130 Å) using the following conditions: mobile phase A was water/formic acid (99.9:0.1, v/v), mobile phase B was acetonitrile/formic acid (99.9:0.1, v/v), the gradient program flow rate was set at 300 μL/min throughout the experiment. The initial composition of the mobile phase was 97% A and 3% B, which was linearly changed to 20% A and 80% B in 7 min and then changed to 2% A and 98% B in 8 min. After maintaining this ratio for 5 min, it was then changed to 100% B in 1 min and held for 3 min. The column temperature was maintained at 45 °C. A 5-μL sample was injected into the column, and the autosampler temperature was set at 4 °C.

Mass spectrometry detection was performed using a mass spectrometer (Waters Q-TOF Premier), equipped with an electrospray ionization (ESI) source in both positive and negative-ion modes. The MS instrument parameters were as follows: the mass scan range was from 0 to 1000 m/z, the MS acquisition rate was 0.3 s with a 0.02-s inter-scan delay, and high-purity nitrogen was used as the nebulizer and drying gas. The nitrogen drying gas was at a constant flow rate of 450 L/h. The source and desolvation temperatures were set at 120 °C and 350 °C, respectively. The capillary voltage was set at 3.0 kV, and the sampling cone voltage was set at 40.0 V in negative mode or 50.0 V in positive mode. Argon was used as the collision gas, and the collision energy was set to 5.0 eV. MS/MS analysis was performed with the mass spectrometer set at different collision energies, ranging from 30 to 80 eV, according to the stability of the individual metabolites.

The time-of-flight analyzer was used in 'V' mode and was tuned for maximum resolution (>10,000 resolving power at m/z 554.2771 in negative ion mode or at m/z 556.2771 in positive ion mode). The instrument was calibrated with sodium

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