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Metabolomic analysis of cholestatic liver damage in mice

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ABSTRACT

Cholestasis is characterized by the obstruction of bile duct, including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). The complicated etiology and injury mechanism greatly limits the development of new drugs for its treatment. To better understand the mechanism of cholestatic liver damage, ultra-performance liquid chromatography-linked electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and multivariate data analysis were used to determine the metabolic changes in three recognized mouse cholestasis models. The cholestatic liver damage was generated by alphanaphthyl isothiocyanate (ANIT), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) and lithocholic acid (LCA). The results indicated that the levels of bile acids were commonly increased in plasma of three mouse cholestasis models, while arginine was decreased. The level of plasma glutathione was decreased in ANIT- and LCA-induced intrahepatic PBC and PSC, respectively. But, the liver glutathione was decreased in DDC induced extrahepatic PSC. The level of plasma phospholipids was elevated in ANIT and DDC models, whereas that was depleted in LCA model. And protoporphyrin IX was significantly increased in the liver of DDC model. These metabolomics data could potentially distinguish the metabolic differences of three types of cholestasis, contributing to the understanding of the potential mechanism of cholestatic liver damage.

1. Introduction

Cholestasis is mainly characterized by disturbance of bile secretion, intake and flow, resulting in accumulation of bile constituents in liver (van Golen et al., 2017). Multiple factors can cause cholestasis, including drug-induced liver injury (DILI), inheritance (progressive familial intrahepatic cholestasis), and disease (sepsis, cholangiolithiasis) (Nguyen et al., 2014). Without timely treatment, cholestasis would further be developed into hepatic failure, fibrosis and cirrhosis. Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are two commonly recognized types of cholestatic liver disease (Ishihara et al., 2009; Nguyen et al., 2014). Statistical data indicate that the prevalence of PBC is 7–402 cases per million people in Europe, while it was 400–500 cases per million in Asian (Liu et al., 2010; Poupon, 2010). For PSC, the prevalence was 16.2 cases per million people, and more than 50% patients need liver transplantation 10–15 years after diagnosis (Lindkvist et al., 2010). Due to the complicated etiology and injury mechanism of cholestasis, it greatly limits the development of new drugs for its treatment. Currently, only two drugs, ursodeoxycholic acid (UDCA) and obeticholic acid (OCA), approved by U.S. Food and Drug Administration (FDA) were applied to treat cholestasis in clinic. However, some patients exhibited tolerance for monotherapy of UDCA. Thus, OCA became a new supplementary agent for patients without response to UDCA therapy (Chascsa et al., 2017). However, there is still a lack of understanding with regards to the exact mechanism of cholestatic liver damage.

Diagnose of cholestasis requires chemical analysis combined with ultrasonography or liver biopsy, and it is difficult to diagnose in early stage without invasive liver biopsy. Generally, chemical analyses of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are the indicators for liver injury, while alkaline phosphatase (ALP), γ-glutamyltransferase (γ-GGT) and total bilirubin (TBIL) are considered to be indicators for cholestasis (Luo et al., 2014). However, these indicators show some limitations in sensitivity and specificity (Kremer et al., 2015; Luo et al., 2014; Yamazaki et al., 2013). Therefore, non-invasive and sensitive diagnostic approaches for cholestasis need to be further developed. Metabolomics can systematically reveal changes in endogenous metabolites induced by genetic variation or external stimuli on organism. Metabolomic technology has been regarded as a potential platform for biomarker discovery, therapeutic agent

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exploration and clinical diagnostics (Fang and Gonzalez, 2014; Hicks et al., 2015; Huang et al., 2014; Idle and Gonzalez, 2007; Wu et al., 2014). Previous studies have reported that metabolomics technology has been successfully applied to identify biomarkers in animal model of cholestasis and intrahepatic cholestasis of pregnancy (Abdel-Aziz and Mahmoud, 2017; Ishihara et al., 2009). The metabolomic characterization of hepatobiliary diseases has been summarized, including cholestasis, liver transplantation, and acute hepatotoxicity in animal models (Beyoglu and Idle, 2013), shedding new light on the systems pathology of the liver.

The cholestatic liver damages induced by alpha-naphthyl isothiocyanate (ANIT), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) and lithocholic acid (LCA) in mice are three classical models for PBC and PSC (Fickert et al., 2014; Pollheimer and Fickert, 2015; Yang et al., 2017). In this study, in order to better understand the mechanism of cholestasis, the metabolic changes in these three cholestasis models were profiled by ultra-performance liquid chromatography-linked electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS)-based metabolomics. The results indicated that the levels of bile acids were commonly increased in plasma of three mouse cholestasis models, while arginine was decreased. The level of plasma glutathione was decreased in ANIT- and LCA-induced intrahepatic PBC and PSC, respectively, while that of liver glutathione was decreased in DDC-induced extrahepatic PSC. The level of plasma phospholipids was elevated in ANIT and DDC models, while that was depleted in LCA model. And liver protoporphyrin IX was significantly increased in DDC model. This study distinguished the metabolic differences of three types of cholestasis, contributing to the understanding to the potential mechanism of different types of cholestasis.

2. Materials and methods

2.1. Chemicals

DDC, ANIT, taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), taurodeoxycholic acid (TDCA), taurohyodeoxycholic acid (THDCA), taurochenodeoxycholic acid (TCDCA), taurolithocholic acid (TLCA), phenylalanine, tyrosine, proline, valine, histidine, arginine, glutamic acid, protoporphyrin IX (PPIX), lysophosphatidylcholine 16:0, 18:0 (LPC 16:0, 18:0), myristoylcarnitine (14:0-carnitine), palmitoylcarnitine (16:0-carnitine), 4,7,10,13,16,19-docosahexaenoic acid (DHA), L-glutathione reduced (GSH) and 5′-(methylthio)adenosine (MTA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Tauro-beta-muricholic acid (T-β-MCA) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Tauroursodeoxycholic acid (TUDCA) and LCA were provided from Medchemexpress (Monmouth Junction, NJ, USA). Tauro-alpha-muricholic acid (T-α-MCA) were purchased from Steraloids (Newport, RI). Lysophosphatidylethanolamine 16:0, 18:0 (LPE 16:0, 18:0) were provided by Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). All the other chemicals and solvents were of the highest grade commercially available.

2.2. Animals studies

Male C57BL/6 mice (6–8 weeks old) were supplied by Slaccas Laboratory Animal Co., LTD. (Hunan, China). Mice were housed under temperature and humidity-controlled conditions with a 12 h light/12 h dark cycle. All animal protocols were approved by the institutional animal care and use committee at the Kunming Institute of Botany, Chinese Academy of Sciences. Thirty mice were randomly divided into six groups: (1) control of ANIT (ANIT-C); (2) ANIT; (3) control of DDC (DDC-C); (4) DDC; (5) control of LCA (LCA-C); (6) LCA. ANIT dissolved in corn oil was given at a single dose of 75 mg/kg by gavage and mice were sacrificed 48 h after treatment (Fang et al., 2017; Tang et al., 2016). The administration of DDC was slightly modified on the previous reports (Dai et al., 2017; Fickert et al., 2007). DDC dissolved in corn oil was given at an oral dose 100 mg/kg for seven consecutive days, and mice were euthanized 24 h after the last dose of DDC. LCA dissolved in corn oil was intraperitoneal administered at dose of 125 mg/kg twice a day for four days consecutively (Beilke et al., 2009; Owen et al., 2010) and sacrificed 24 h after the last dose of LCA. The mice treated with corn oil were used as the control groups. All the mice were sacrificed by CO_2 inhalation. Subsequently, mice plasma and liver samples were collected for biochemical assay, histopathology and metabolomic analysis. Biochemical assay included ALT, AST and ALP. Part of liver tissue were stained with hematoxylin and eosin (H&E) for histopathology using the method described previously (Hu et al., 2018) and the others were stored at −80 °C.

2.3. Sample preparation

Plasma and liver samples were prepared as the following procedure. In brief, 10 μL plasma was mixed with 190 μL 67% acetonitrile/water (containing chlorpropamide 5 μM). After vortexed twice for one min, the samples were centrifuged at 18,000 g for 20 min at 4 °C. Liver samples (50 mg) were homogenized in 500 μL ice cold 50% acetonitrile/water (containing chlorpropamide 5 μM) at 6500 Hz, 15 s for 3 cycles using homogenizer (Precellys, Bertin Technologies, France). After shaking for 20 min at room temperature, the samples were centrifuged 18,000 g for 20 min at 4 °C. The supernatant (150 μL) were diluted with 150 μL cold acetonitrile, then vortexed and centrifuged. Finally, 5 μL of the supernatant was injected for UPLC-QTOF-MS analysis.

2.4. UPLC-QTOF-MS analysis

Metabolites of plasma and liver samples were analyzed by Agilent 6530 quadrupole time-of-flight mass spectrometer (Agilent, Santa Clara, CA, USA) which coupled with a 2.1 × 100 mm 1.8 μm XDB-C18 column (Agilent, Santa Clara, CA, USA). Mobile phase A was water containing 0.01% formic acid and mobile phase B was acetonitrile containing 0.01% formic acid. With constant flow rate 0.3 mL/min, the elute gradient was set as follow: 2–98% B from 0 to 12 min, kept 2 min then returned to 2% B and maintained to 16 min. Data was collected under both positive and negative mode. For HILIC mode, sample analysis was performed on a 2.1 × 100 mm, 2.7 μm Poroshell 120 HILIC column (Agilent, Santa Clara, CA, USA). The mobile phase and flow rate were similar to RPLC. The gradient ranged from 98% to 65% B at 0.5–3 min, next decreased to 40% at 8 min, then returned to 2% for 3 min and increased to 98% to 16 min. Data was collected within *m/z* range from 50 to 950 under both modes.

2.5. Multivariate data analysis

The mass spectrometric data were exported to a CSV file by Agilent MassHunter Profinder B.06.00 software (Agilent, Santa Clara, CA, USA), which contained ion *m/z* ratio, retention time (Rt) and relative peak intensity. The data were normalized using internal standard and analyzed by unsupervised principal components analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) by SIMCA-P + 13.0 (Umetrics, Kinnelon, NJ). Metabolites were identified by comparing the Rt, MS/MS fragments with authentic standards, reported references or public databases, including METLIN (http://metlin.scripps.edu/) and HMDB (http://www.hmdb.ca/).

2.6. Statistical analysis

Mean ± SEM was calculated for all experiment values. The statistical analysis was performed using two-tailed Student's *t*-test by GraphPad Prism 6.0 (San Diego, CA), and p value < 0.05 was considered as significant.