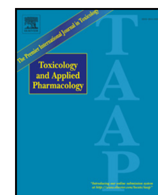
Toxicology and Applied Pharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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



Increased serum bile acid concentration following low-dose chronic administration of thioacetamide in rats, as evidenced by metabolomic analysis

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A R T I C L E   I N F O

Article history:
Received 23 March 2015
Revised 18 July 2015
Accepted 20 July 2015
Available online xxxx

Keywords:
Thioacetamide
Metabolomics
Bile acids
Hepatotoxicity
Transporter
Enterohepatic circulation

A B S T R A C T

A liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS)-based metabolomics approach was employed to identify endogenous metabolites as potential biomarkers for thioacetamide (TAA)-induced liver injury. TAA (10 and 30 mg/kg), a well-known hepatotoxic agent, was administered daily to male Sprague–Dawley (SD) rats for 28 days. We then conducted untargeted analyses of endogenous serum and liver metabolites. Partial least squares discriminant analysis (PLS-DA) was performed on serum and liver samples to evaluate metabolites associated with TAA-induced perturbation. TAA administration resulted in altered levels of bile acids, acyl carnitines, and phospholipids in serum and in the liver. We subsequently demonstrated and confirmed the occurrence of compromised bile acid homeostasis. TAA treatment significantly increased serum levels of conjugated bile acids in a dose-dependent manner, which correlated well with toxicity. However, hepatic levels of these metabolites were not substantially changed. Gene expression profiling showed that the hepatic mRNA levels of *Ntcp*, *Bsep*, and *Oatp1b2* were significantly suppressed, whereas those of basolateral *Mrp3* and *Mrp4* were increased. Decreased levels of Ntcp, Oatp1b2, and Ostα proteins in the liver were confirmed by western blot analysis. These results suggest that serum bile acids might be increased due to the inhibition of bile acid enterohepatic circulation rather than increased endogenous bile acid synthesis. Moreover, serum bile acids are a good indicator of TAA-induced hepatotoxicity.

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1. Introduction

Thioacetamide (TAA), a thiono-sulfur containing compound, induces both acute hepatic failure and chronic hepatic failure. A single dose of TAA causes centrilobular hepatic necrosis; prolonged TAA exposure results in bile duct proliferation and liver cirrhosis, which is histologically similar to the pathology observed in human viral hepatitis infections (Ingawale et al., 2014; Yeh et al., 2004). TAA is frequently used as a model hepatotoxin, as it selectively causes liver damage, including cirrhosis, fibrosis, and hepatic necrosis/apoptosis (Li et al., 2002; Okuyama et al., 2005).

TAA undergoes extensive metabolism in the liver, and less than 1% of the dose is excreted unchanged (Rees et al., 1966). TAA-induced toxicity results from its bioactivation via cytochrome P450 (CYP) and flavin-containing monooxygenases (Hajovsky et al., 2012). Further, CYP2E1 plays a major role in the generation of toxic intermediates (Wang et al., 2000). Metabolic activation of TAA then leads to the formation of reactive metabolites, which are represented by sulfin or sulfen metabolites derived from thioacetamide S-oxide (Sarma et al., 2012) and reactive oxygen species (ROS) generated as intermediates (Chilakapati et al., 2007). Mechanisms responsible for the development of TAA-induced liver injury have been studied intensively. It is generally accepted that reactive intermediates can covalently bind to cellular macromolecules and/or induce oxidative stress. ROS generated following TAA administration can cause lipid peroxidation and glutathione depletion (Sanz et al., 2002). In addition, TAA also induces calcium mobilization from intracellular stores (Diez-Fernandez et al., 1996). Both ROS and calcium presumably activate multiple reactions related to cellular damage or proliferation (Brookes et al., 2004). Increased ROS formation and disruption of calcium homeostasis can increase inner mitochondrial membrane permeability, disrupt mitochondrial membrane potential, and inhibit mitochondrial respiration (Bernardi et al., 2001).

Recent studies showed that metabolomic approaches are useful in elucidating toxic mechanisms and predicting chemical-induced toxicity (Bouhifd et al., 2013). Analytical techniques used in metabolomic

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http://dx.doi.org/10.1016/j.taap.2015.07.016
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Please cite this article as: Jeong, E.S., et al., Increased serum bile acid concentration following low-dose chronic administration of thioacetamide in rats, as evidenced by metabo..., Toxicol. Appl. Pharmacol. (2015), http://dx.doi.org/10.1016/j.taap.2015.07.016

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studies include nuclear magnetic resonance spectroscopy (NMR), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass spectrometry (LC–MS). Among them, high-resolution LC–MS is a promising tool for generating metabolomics data that can enable identification of potential biomarkers involved in drug-induced toxicity (Fang and Gonzalez, 2014). Various potential biomarkers were identified and proposed to aid in the prediction of hepatotoxicity and nephrotoxicity caused by acetaminophen (Chen et al., 2009), gemfibrozil (Liu et al., 2014), isoniazid (Cheng et al., 2013), monocrotaline (Conotte and Colet, 2014), and flutamide (Choucha Snouber et al., 2013).

In the present study, we used LC/MS-based metabolomics analysis to evaluate changes in endogenous metabolites found in rat serum and the liver after 28-day TAA treatment. We aimed to identify potential biomarkers to aid in the prediction of TAA-induced hepatotoxicity and to elucidate the mechanisms involved in toxic responses. We also used conventional clinical chemistry and histopathology to concurrently investigate the toxic threshold and toxicity biomarkers associated with TAA-induced hepatotoxicity. Finally, the mechanism of TAA-induced disruption of bile acid metabolism was characterized.

2. Materials and methods

2.1. Chemicals and reagents

TAA and bile acid standards including cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), taurolithocholic acid (TLCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), and glycoursodeoxycholic acid (GUDCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholic acid-d_5, as an internal standard was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). High performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA). All other chemicals used in this study were of the highest grade commercially available.

2.2. Animal study and sample collection

Animal studies were conducted in accordance with policies set forth by the Korea Institute of Toxicology Institutional Review Board. Male Sprague–Dawley (SD) rats (6–7 weeks of age, 200–220-g body weight) were obtained from Orient Bio (Seongnam, Korea) and were acclimated for 1 week. The animal quarters were maintained at 23 ± 3 °C and 50% ± 10% relative humidity. A 12-h light and dark cycle was maintained, with a light intensity of 150–300 lx. Rats were provided with a commercial pellet diet and tap water ad libitum. Two oral doses of 10 mg/kg (n = 10) or 30 mg/kg (n = 10) TAA were administered to animals daily for 28 consecutive days. The control group animals (n = 10) were orally treated with the same volume of vehicle (0.5% carboxymethyl cellulose). The rats were overnight fasted prior to sacrifice and were anesthetized with inhaled isoflurane 24 h after the last dosing. Blood samples were collected via the posterior vena cava and liver samples were harvested. The blood samples were placed at room temperature for 2 h, and then serum samples were obtained after centrifugation. Clinical chemistry analysis was performed on serum sample aliquots using an automated chemistry analyzer (Toshiba Co., Japan). The remaining serum samples were stored at −80 °C until use in metabolomic and quantitative bile acid analyses. For histological analysis, small blocks of the left lateral lobes of the liver were immediately fixed in 10% formalin and then embedded in paraffin. Sections (5 μm) were then stained using hematoxylin and eosin (H&E). Histopathologic categories were scored on the basis of the pathologist's impression. The scoring system used in all categories consisted of a scale of 1 through 4 (1 = no abnormalities, 2 = mildly abnormal, 3 = moderately abnormal, and 4 = markedly abnormal). Other liver samples were submerged into liquid nitrogen and stored at −80 °C until use in whole genome microarray and metabolomics analyses.

2.3. Untargeted metabolomics analysis

For the serum samples, three volumes of acetonitrile were added to the 50-μL serum sample, the mixture was vortexed, and then centrifuged at 13,200 rpm for 10 min at 4 °C. The supernatant was removed, and then a 3-μL aliquot of the supernatant was injected into the HPLC column. For the liver tissue, approximately 100 mg of tissue was homogenized with 300 μL of cold water/methanol (50:50) by ultrasonication (10 s × 3 cycles), and then centrifuged at 13,200 rpm for 10 min at 4 °C. The supernatant (150 μL) was evaporated to dryness under a stream of N2, and then dissolved in 150-μL water/methanol (90:10) before analysis. Analysis of all samples was performed on an Agilent 6530 quadrupole time-of-flight mass spectrometer (Agilent, Santa Clara, CA, USA) coupled with an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA, USA). The separation was performed with a Luna C18 column (100 × 2.1 mm, 3 μm; Phenomenex, Torrance, CA, USA) using a gradient mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.2 mL/min. The initial composition of mobile phase B was 10%, increased to 40% in 5.0 min, then increased to 95% in 15 min and maintained for 3.0 min, followed by re-equilibration to the initial condition for 7 min. The total run time was 30.0 min. The column and autosampler temperatures were maintained at 35 °C and 4 °C, respectively. The injection volume was 3 μL. Electrospray ionization (ESI) was performed in both the positive ion mode and negative ion mode. The sheath gas flow rate was set at 11 L/min. The drying gas flow rate was set at 11 L/min at a temperature of 350 °C. The nebulizer temperature was maintained at 350 °C. The capillary voltage was set at 4000 V in the positive mode and −4000 V in the negative mode, and the fragmentor was set at 175 V. Data were acquired in the scan range from *m/z* 50 to 1,000 in the centroid mode. A reference compound ($C_{18}H_{18}O_6N_3P_3F_{24}$; $[M + H]^+ = 922.0098$ and $[M + formate]^- = 966.0007$) was used to correct the mass during the analysis. Auto MS/MS analysis was performed for peak identification and the collision energy was set at 30 eV.

2.4. Data processing

MS data were obtained according to the following parameters: retention time range, 0.5 to 18 min; mass range, *m/z* 100 to *m/z* 1,000; and extracted ion chromatogram window, 0.02 Da. The data were then converted to mzXML format files using conversion software (download, http://proteowizard.sourceforge.net). The data were processed using the XCMS package in R statistical software 3.1.0 (http://cran.r-project.org/) for peak detection, alignment, and integration. The optimized XCMS parameters were as follows: method = Cent Wave, signal-to-noise threshold (sn thresh) = 10, maximum *m/z* deviation tolerance (ppm) = 30, peak width = (10, 30), bandwidth (bw) = 10, and integration method = 1 (default). Other parameters were set to the default. The peak was annotated for isotopes and adducts using the CAMERA package. The matrix (m/z–retention time–intensity pairs) was generated and exported to a CSV file. Microsoft Excel 2010 was used to remove isotopes and filter the data according to the 80% rule and a noise cut-off value of 10,000. The data were analyzed using the SIMCA-P11.5 software package (Umetris AB, Umea, Sweden) for multivariate statistical analysis; all variables were Pareto-scaled [$(X - \overline{X})/\sqrt{SD}$, $\overline{X}$; Mean, SD; standard deviation]. Principle component analysis (PCA) was performed to visualize any patterns and groupings, and partial least squares-discriminant analysis (PLS-DA) was performed to determine separate influential variables between the treated groups and controls based on their variable importance in projection (VIP).

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