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# Investigation of nonalcoholic fatty liver disease-induced drug metabolism by comparative global toxicoproteomics

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) includes conditions such as steatosis, non-alcoholic steatohepatitis, and ultimately hepatocellular carcinoma. Although the pathology of NAFLD is well-established, NAFLD-induced drug metabolism mediated by cytochrome P450 (CYP) in the liver has remained largely unexplored. Therefore, we investigated NAFLD-induced drug metabolism mediated by CYP by quantitative toxicoproteomics analysis. After administration of a methionine-choline deficient (MCD) diet to induce development of NAFLD, tandem mass tags-based liquid chromatography-tandem mass spectrometry analysis was conducted to investigate the dynamics of hepatic proteins. A total of 1295 proteins were identified, of which 934 were quantified by proteomic analysis. Among these proteins, 21 proteins were up-regulated and 51 proteins were down-regulated by the MCD diet. Notably, domain annotation enrichment using InterPro indicated that proteins related to CYPs were significantly decreased. When we investigated CYP activity using *in vivo* and *in vitro* CYP cocktail assays, most CYPs were significantly decreased, whereas CYP2D was not changed after administration of the MCD diet. In conclusion, we identified significantly altered levels of CYPs and their activities induced by the MCD diet and confirmed the NAFLD-induced drug metabolism by pharmacokinetic analysis.

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a histological spectrum from hepatocellular steatosis with minor inflammation to non-alcoholic steatohepatitis (NASH) with inflammation and ballooning liver tissue. NASH tends to progress to more serious diseases such as irreversible cirrhosis and hepatocellular carcinoma, leading to mortality (Dowman et al., 2010). NAFLD pathogenesis is caused by the accumulation of lipids within hepatocytes, mostly in the form of triglycerides (TG) (Angulo, 2002; Gaggini et al., 2013). The cause of lipid accumulation may be related to problems in uptake, synthesis, degradation, or secretion in hepatic lipid metabolism resulting from insulin resistance (Angulo, 2002; Giulio Marchesini et al., 1999). NAFLD is considered as a metabolic syndrome in the liver linked to obesity, type 2 diabetes mellitus, and hyperlipidemia (Younossi et al., 2018). The prevalence of NAFLD has increased rapidly in parallel with these diseases; particularly, the global obesity prevalence will reach 18% in men and surpass 21% in women by 2025 (Collaboration, 2016). Among the worldwide patients with NAFLD, 51% suffer from obesity, 22.5% from type 2 diabetes, and 42% from metabolic syndromes (Younossi et al., 2016).

As is evident in many previous reports, NAFLD is the most common chronic liver disease among adults and children (Abd El-Kader and El-Den Ashmawy, 2015). The patients are administered a variety of medications such as anti-diabetic drugs, anti-hyperlipidemia drugs, and weight control aids for treatment. NAFLD is a liver-specific disease, and thus may modulate the activity of metabolic enzymes. However, the NAFLD-induced drug metabolism and cytochrome P450 (CYP)-modulating effect of NAFLD remain unclear. A few studies of the modulatory effect of NAFLD on metabolic enzymes have been reported. For example, the mRNA levels of CYPs were investigated in mice fed a high-fat and high sucrose diet, and the enzyme-kinetic and pharmacokinetic

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parameters for CYP2B1-mediated bupropion metabolism were recently examined (Chiba et al., 2016; Cho et al., 2016). However, studies of NAFLD-induced drug metabolism are lacking.

Mice fed the methionine-choline deficient (MCD) diet is a commonly used animal model for NASH and NAFLD and induces steatosis, oxidative stress, inflammation, and fibrosis (Hebbard and George, 2011; Jha et al., 2014a; Jha et al., 2014b). Methionine is an amino acid involved in the synthesis of proteins, *S*-adenosylmethionine, and glutathione (Mato et al., 2008; Mato et al., 2013). Methionine deficiency can cause oxidative stress, inflammation, and fibrosis (Caballero et al., 2010; Jha et al., 2014b). Additionally, choline is essential for the *de novo* synthesis of phosphatidylcholine, which is required for TG export *via* very low-density lipoprotein secretion. Ultimately, a choline-deficient diet can cause liver steatosis (Caballero et al., 2010; Vance, 2008).

Proteomic analysis can generate abundant information on individual proteins involved in biological responses and is an irreplaceable tool for studying the responses of living organisms to their environments (Deracinois et al., 2013). The combination of differential expression proteomics with tandem mass tags (TMT) has been used to investigate proteins in various studies for biological discovery, quantification, and protein analysis (Liu et al., 2015; Wang et al., 2015a; Wang et al., 2015b). As a powerful quantitative proteome analysis technique, TMT-based liquid chromatography (LC)-tandem mass spectrometry (MS/MS) adopts stable isotope labeling strategies of proteins or peptides for measurement and allows identification of more proteins, as well as provides more reliable quantitative information compared to traditional two-dimensional gel electrophoresis (2-DE) analysis (Aebersold and Mann, 2003). However, very few studies using proteomic analysis to study the modulation of drug metabolism in NAFLD have been reported (Xia et al., 2017; Zhonghua et al., 2015).

Thus, we developed an NAFLD model in male C57BL/6J mice after feeding of the MCD diet for 4 weeks. Based on global comparative proteomic analysis, we investigated the relative levels of 934 quantified proteins in the liver between the normal and MCD diet groups, including bioinformatics analysis using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. We identified significantly changed metabolic enzymes, particularly CYPs. The enzyme activities and pharmacokinetic parameters of 5 CYP isoforms were determined in a cocktail assay *in vitro* and *in vivo* to predict NAFLD-induced drug interactions.

## 2. Material and methods

### 2.1. Animals

Male 5-week-old C54BL/6 N mice were purchased from Orient Co. (Seongnam, Korea) and randomly housed with 4 mice per cage. Before the experiments, the mice were allowed one week of acclimation in a controlled environment (relative humidity: 60%, temperature: maintained at 25 °C) under a 12-h/12-h light/dark cycle, with free access to standard rodent chow and tap water. We fed the mice either a normal diet (ND) (n = 13) or MCD (n = 13) (Central Lab. Animal Inc., Seoul, Korea) for 4 weeks. Mice from each group were sacrificed to obtain liver tissues and blood samples after overnight fasting. Aliquots of liver tissue were prepared for all samples for use in subsequent analyses. All animal procedures were conducted in compliance with the guidelines issued by Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and this study was approved by the Institutional Review Board of Kyungpook National University (KNU-2017-0089-1).

### 2.2. Liver sample preparation for proteomic analysis

For proteomic profiling, 10 mg mouse liver from three mice in the ND and MCD groups were used to extract protein with 1% SDS sample buffer containing a protease inhibitor cocktail (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein was reduced with 5 mM dithiothreitol at 56 °C for 30 min and alkylated with 15 mM iodoacetamide at 25 °C for 30 min in the dark. After reduction and alkylation, the three mouse livers were pooled. For protein precipitation, 200 μg of protein was added to 10% of trichloroacetic acid and incubated for 4 h at 4 °C and the pellets were washed twice in ice-cold acetone. The protein in 50 mM triethyl ammonium bicarbonate was then digested by sequencing-grade modified trypsin (Promega, Madison, WI, USA) for 16 h at 37 °C. The ratio of enzyme to protein was 1:50. The digested peptides were labeled with the 2-plex\TMT reagent (Thermo Fisher Scientific Inc.) according to the manufacturers protocol. Briefly, 40 μg of peptide samples was labeled by adding 0.4 mg of TMT Label Reagent (ND, 126; MCD diet, 127) and incubated for 1 h at 25 °C. The peptides were then high-pH fractionated (Thermo Fisher Scientific Inc.) with eight different buffers according to the manufacturer's protocol. Briefly, acetonitrile (ACN) solution (in 0.1% triethylamine) at eight different percentage concentrations was used to elute the TMT-labeled peptides. All fractioned samples were cleaned with C18 Ziptips (Millipore, Billerica, MA, USA) according to the manufacturer's protocols.

### 2.3. Nano LC-MS analysis for proteomic analysis

TMT-labeled peptides (5 μg) were subjected to LC-MS/MS with a hand-made C12 reverse-phase analytical column (Proteo C12 4-μm beads, 90-Å pore size, Phenomenex, Torrance, CA, USA). A 60-min gradient was used at a flow rate of 300 nL/min: from 3 to 21% solvent B (0.1% formic acid and 10% water in acetonitrile) for 50 min, and 21–90% solvent B for 3 min, using the Ekigent nanoLC (SCIX, Redwood City, CA, USA). The temperature of the column was maintained at 30 °C. The electrospray voltage was 2.0 Kv and the mass spectrometer was operated in data-dependent MS/MS mode. MS precursor scans ($m/z$ range of 300–1800 Th) were acquired at an automated gain control target value of $1.0 \times 10^6$ and resolution of 60,000. The MS/MS data for up to the 10 most abundant ions were acquired in data-dependent mode using higher-energy collisional dissociation at a normalized collision energy of 40, with a fixed first mass of 100 Th at a resolution of 7500, automated gain control target value of $1.0 \times 10^6$, and maximum injection time of 100 ms. The peptides were ionized using a nanospray ionization source, followed by MS/MS in a LTQ Velos-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) linked online to the UPLC.

### 2.4. Protein identification and quantification

Peptides were identified using MaxQuant 1.5 (Han et al., 2017) with a precursor mass error of 10 ppm, selected monoisotopic mass, and fragment ion mass error of 20 ppm. Tandem mass spectra were searched against the UniProtKB/Swiss-Prot Mouse database (51,444 proteins) concatenated with the reverse decoy database and protein sequences of common contaminants. The protein was digested with trypsin allowing two missed cleavages. Cysteine carbamidomethylation (57.021 Da) and TMT-2 plex on the peptide N-terminus and lysine (229.163 Da) were searched as fixed modifications. Methionine oxidation (15.995 Da) and protein N-terminus acetylation (42.011 Da) were searched as variable modifications. A decoy search was performed, peptides were filtered using a false-discovery rate (FDR) of 0.01, and reverse and potential contaminant proteins were removed from the total identified proteins. To quantify proteins by TMT labeling, reporter ions were calculated by the TMT-2 plex method using MaxQuant 1.5. All other parameters in MaxQuant were set to default values. Additionally, quantified proteins were defined as upregulated at ratios of > 1.5 and down-regulated at ratios below 0.666.

### 2.5. Bioinformatics

The identified proteins were annotated using KEGG, InterPro, and GO, which consist of GO molecular function, GO biological process, and