METABOLOMIC INVESTIGATION OF ALCOHOL-INDUCED FATTY LIVER

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ABSTRACT

Alcohol abuse is an important health issue in the world. While the adverse effect of alcohol can spread to various of tissues, the most common ailment associated with alcohol abuse is alcoholic liver disease (ALD) which is characterized by progressive manifestations from fatty liver to hepatic cirrhosis. As the early stage of ALD is reversible, numerous efforts have been undertaken to understand the molecular mechanisms of ALD and identification of reliable biomarkers. It is suggested that nutritional effects and the toxic effects of alcohol both contribute to the pathogenesis of ALD and may interact with each other. This offers an alternative aspect for biomarker seeking as focuses on the influence of alcohol on nutritional small molecule metabolome which can indicate the *in vivo* change caused by alcohol consumption. The newly developed metabolomics-based technical platform has provided a robust tool to identify and characterize new small-molecule biomarkers of biochemical processes and pathogenesis of ALD.

In my study, the influence of ethanol on small-molecule metabolome and the role of cytochrome P450 2E1 (CYP2E1) in ethanol-induced hepatotoxicity were investigated using liquid chromatography-mass spectrometry (LC-MS) based metabolomics platform and *Cyp2e1*-null mouse model. The results suggested CYP2E1 contributes to ethanol-induced toxicity. Metabolomic analysis revealed that the level of *N*-acetyltaurine (NAT) in urine increases dramatically after ethanol consumption. Further *in vivo* and *in vitro* analysis indicated that ethanol-induced NAT biosynthesis is mainly caused by a novel

reaction between taurine and excessive acetate produced by ethanol metabolism. Overall, our study confirmed the role of CYP2E1 in alcohol-induced liver injury and identified NAT as a novel metabolite of ethanol that can function as the potential biomarker of hyperacetatemia.

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[1] **Key Words:** Alcohol; Alcoholic liver disease; Alcohol metabolism; Nutrients; Metabolomics;

[1] **Abbreviations**: ALD, alcoholic liver disease; EtG, ethyl glucuronide; EtS, ethyl sulfate; ADH, alcohol dehydrogenase; MEOS, microsomal ethanol-oxidizing system; ALDH, aldehyde dehydrogenase; ACS, acetyl-CoA synthetase; NAD, nicotinamide adenine dinucleotide; NADH; reduced NAD; CYP2E1, cytochrome P450 2E1; ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, glutathione; NMR, nuclear magnetic resonance; MS, mass spectrometry; gas chromatography; LC, liquid chromatography; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; TOF, time-of-flight; FT, Fourier transform; MDA, multivariate data analysis; RT, retention time; PC, principle components; PCA, principal components analysis; PLS, partial least squares; OPLS, orthogonal partial least squares; SIMCA, soft-independent modeling of class analogy; PLS-DA, partial least squares-discriminant analysis;

1.1 ALCOHOLIC LIVER DISEASE

1.1.1 Alcohol abuse

Alcoholism and alcohol abuse is defined as the regular and excessive use of alcohol that is associated with concomitant physical, emotional, and social problems (Lowenfels, 2000). In the past 20th century, alcohol abuse has emerged as a major problem with global health implications. Thirteen percent of the adult population in the United States has a history of alcohol dependence or alcohol abuse, and the 12-month prevalence of alcohol dependence is between 4% and 5% (Grant, 1997).

Alcohol abuse and dependence place a heavy burden on the society as the costs of medical complications related to alcohol abuse in the United States are estimated to be nearly $100 billion per year (*3*). In addition to its economic impact, alcoholism and alcohol abuse lead to significant medical morbidity and mortality. About 10% of alcohol consumers will at some point experience serious health problems related to their drinking habit (*4*). Alcoholism and alcohol abuse are responsible for 105, 000 deaths per year in the United States (*5*).

Alcohol abuse is associated with increased risk for a variety of medical problems including hepatic cirrhosis, cardiomyopathy, various cancers, infectious diseases, fetal abnormalities, and neurologic complications including dementia (*6*, *7*).

1.1.2 Alcoholic liver disease

As a major cause of liver disease, alcoholic liver disease (ALD), is the hepatic manifestations of alcohol overconsumption (*8*). These manifestations are often grouped into three histological stages of ALD, including fatty liver or steatosis featured by the accumulation of fatty acids in liver cells, alcoholic hepatitis characterized by the inflammation of hepatocytes, and hepatic cirrhosis, which is the late stage of serious liver disease marked by inflammation (swelling), fibrosis (cellular hardening), reduced detoxification of chemicals, scarring and necrotic cell death (*9*). The clinical symptoms of alcohol-related liver injury from steatosis to cirrhosis and even to liver cancer are not necessarily progressed as sequential events. In fact, multiple stages of ALD may be present simultaneously in a given individual (*10, 11*).

Fatty liver is the remarkable characteristic of the first stage in ALD pathogenesis. Up to 90% of alcoholics who drink more than 60 g/day of alcohol develop fatty liver (*12*). However, this condition is generally asymptomatic and self-limited, and may be entirely reversible with abstinence (*13*). Hepatic fibrosis which is characterized by the excess formation of fibrous connective tissue in the liver is believed to start in the perivenular area and occur in 40-60% of the patients who ingest more than 40-80 g/day for an average of 25 years (*14*, *15*). Over a 20-year follow-up period, 5–15% of these patients will develop alcoholic cirrhosis (*16, 17*) which may develop in succession or exist concomitantly with hepatitis and fibrosis.

Like other chemical-induced liver injuries, the development of ALD is dose-dependent and the amount of alcohol ingested is considered the most important risk factor of ALD.

It is suggested that the risk of developing cirrhosis increases with the ingestion of >60-80 g/day of alcohol for 10 years in men, and >20 g/day in women (*18*). However, there are many other factors that can affect the development of liver injury induced by alcohol, including duration, type of alcohol beverages, drinking patterns, gender, ethnicity, obesity, iron overload, concomitant infection with viral hepatitis, genetic factors, and nutritional factors (*19-21*).

1.1.3 Significance of ALD biomarkers

One prominent feature of ethanol-induced chronic toxicities is the gradualism of disease development, i.e. ALD always starts from a reversible stage of hepatosteatosis, and then gradually progresses to the irreversible stages of hepatic fibrosis and cirrhosis, and liver cancer in certain cases (*9*). As the modulations of biochemical pathways and metabolic reactions occur at each stage of ethanol-elicited diseases, examining these events in cellular and molecular levels provides an excellent venue for discovering specific and sensitive biomarkers that can indicate the extent of ethanol abuse and the scope of tissue damage. Generally, current markers of ALD can be divided into three categories: histological biomarkers, protein biomarkers and small molecule markers which can be applied alone or combined in different conditions to provide information for diagnosis or monitoring disease progress. Based on the histological features prominent in the different stages of alcohol-induced liver injury, a liver biopsy is useful to define the stage and severity of the liver disease, especially when other reliable clinical and biochemical indicators are not available (*22*). Established protein biomarkers of ALD include enzymes

whose level can be elevated due to alcohol-induced cell death, such as γ-glutamyltransferase and transaminases (*23, 24*); serum proteins, such as carbohydrate-deficient transferrin in the serum and protein adducts formed by alcohol metabolites, such as acetaldehyde adducts (*23, 25, 26*). Besides histological and protein biomarkers, small-molecule biomarkers whose levels are altered by alcohol consumption have been found (*27*). The small-molecule biomarkers that indicate alcohol abuse and liver injury come from two sources. One is from alcohol metabolism itself, such as ethyl glucuronide (EtG) and ethyl sulfate (EtS) which are two minor metabolites of ethanol and associated with the amount of ethanol exposure (*28, 29*) (detailed in **1.2**). The other is from alcohol-induced disruption of nutrient metabolism (detailed in **1.3**), such as 5-hydroxytryptophol which is a serotonin metabolite elevated after alcohol consumption (*30*). Compared to histology and protein biomarkers, small-molecule biomarkers have clear advantages in specificity, non-invasiveness, convenience and low-cost. In addition, the newly developed metabolomics-based technical platform has provided a robust tool to identify and characterize new small-molecule biomarkers of biochemical processes and pathogenesis (*27*). Since both alcohol metabolism and alcohol-induced interruption of nutrition metabolism, the two sources of small-molecule biomarkers of ALD, lead to the changes in the metabolome, it is reasonable to hypothesize that metabolomic analysis is likely an effect approach to discover novel biomarkers of ALD.

1.2 ALCOHOL METBAOLISM AND LIVER INJURY

Alcohol metabolism, especially the formation of reactive intermediates, is a major

contributing factor in the pathogenesis of ALD. Generally, the liver is the main site of alcohol metabolism. The oxidation of alcohol to acetaldehyde in the liver is primarily initiated through two pathways: the alcohol dehydrogenase (ADH) pathway and the microsomal ethanol-oxidizing system (MEOS) pathway. Catalase-mediated reaction is another pathway mediating the conversion of ethanol to acetaldehyde. However, its role is more important in the extrahepatic tissues, especially in the brain (*31*). Acetaldehyde is a highly unstable compound and quickly forms free radical entities that are highly toxic to cells (*32, 33*). Aldehyde dehydrogenase (ALDH) is responsible for its transformation to less toxic acetate. Acetate can be further catalyzed by acetyl-CoA synthetase (ACS) to generate acetyl-CoA, an important intermediate in the intermediary metabolism. The roles of these metabolizing enzymes in alcohol metabolism and the development of ALD are discussed below and summarized in Figure 1.1.

1.2.1 Alcohol dehydrogenase (ADH)

ADH, which initiates ethanol metabolism and converts ethanol to the toxic substance acetaldehyde, is responsible for low-level ethanol from intestinal flora or moderate alcohol consumption in the liver due to its lower K_m value (*34, 35*). In addition to acetaldehyde, ADH–mediated oxidation of alcohol reduces nicotinamide adenine dinucleotide (NAD) to reduced NAD (NADH), which is an energy carrier in the cell and participate in many essential biochemical reactions (Figure 1.2). The ratio of NAD to NADH is tightly regulated to maintain the redox balance and proper functioning of the cell. When alcohol overconsumption generates excessive amounts of NADH, it may

overwhelm the ability of the hepatocyte to maintain the normal NADH/NAD ratio and lead to metabolic disorders (*36*). For example, elevated levels of NADH can cause abnormally high levels of lactic acid, which in turn reduce the capacity of the kidney to excrete uric acid (*37*). When uric acid accumulates in the body, gout develops, leading to the common clinical observation of gout attacks in ALD patients. Furthermore, an increased NADH/NAD ratio favors synthesis of fatty acids, such as the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate and further to glycerolipids, while suppresses the catabolism of fats in the liver, resulting in the accumulation of fatty acids in the liver and serum (*38*, *39*).

Nutrition status can significantly affect the rate of ethanol oxidation through regulating ADH. It has been showed that low-protein diets reduce the levels of ADH in the liver, resulting in the lower rate of alcohol oxidation in both humans and laboratory animals (*40*). Prolonged fasting also decreases the rate of alcohol oxidation in isolated rat liver cells (*37*). These observations suggest that alcohol is retained longer in malnourished subjects compared to the well–nourished ones. As a result, malnourished alcoholics may also develop higher blood alcohol level that can cause more severe damage to the liver and other organs.

1.2.2 Microsomal Ethanol–Oxidizing System (MEOS)

Compared to the ADH pathway that is mainly responsible for the metabolism of low to moderate amount of alcohol, the MEOS pathway is more important in alcohol metabolism after chronic heavy alcohol consumption. The MEOS has been the subject of

extensive research because it is inducible by long–term alcohol consumption and also involves in the metabolism and elimination of drugs and other xenobiotics from the body (*41*). Therefore, activation of the MEOS after alcohol consumption not only affects alcohol metabolism, but also mediates the interactions between alcohol and those affected xenobiotics.

The MEOS consists of several enzymes located in the microsomal fraction of the liver. The primary component of MEOS is cytochrome P450s, a superfamily of enzymes responsible for the biotransformation of both endogenous compounds, such as lipids and steroidal hormones, and xenobiotics, including drugs, environmental chemicals and alcohol (*42*). Cytochrome P450 2E1 (CYP2E1) has been recognized as the most important P450 involved in alcohol metabolism based on both *in vivo* and *in vitro* studies (*43*). In addition, it is found that the expression of CYP2E1 protein as well as its mRNA is several folds higher in both human and animal studies after chronic alcohol consumption (*44, 45*), while the levels of ADH in the liver did not change following alcohol consumption (*46*). It has been suggested that both the CYP2E1-mediated alcohol metabolism and the induction of CYP2E1 in response to chronic alcohol consumption probably contributes to the development of ALD through the mechanisms elaborated below and summarized in Figure 1.2.

1.2.2.1 Generation of toxic acetaldehyde

Similar to ADH, CYP2E1 converts ethanol to acetaldehyde, a reactive metabolite that can deactivate proteins, increase collagen production, inhibit DNA repair and impair the

electron transport chain and result in liver injury (*47, 48*).

1.2.2.2 Oxygen deficit

After chronic ethanol administration, increased oxidation of ethanol is accompanied by increased total hepatic O_2 utilization required by the CYP2E1 pathway. As a result, the large depletion of oxygen can lead to hypoxic damage in cells and tissues (*49, 50*).

1.2.2.3 Production of reactive species

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been identified as the key components in initiating and possibly sustaining the pathogenic pathways responsible for the progression of ALD (*51*). Alcohol metabolism via CYP2E1 pathway directly contributes to the generation of ROS and RNS. Moreover, excessive NADH produced from alcohol metabolism promotes the production of oxygen radicals (*52*). These reactive species can disrupt mitochondrial function through post-translational modifications to the mitochondrial proteome. Thus may disable the capacity of mitochondria to maintain a normal hepatic ATP level and predispose the liver to permanent damage since the depression of the ATP-requiring anabolic pathways slows the repair and replacement of damaged cellular macromolecules (*53*). In addition, it has been showed that ROS can damage liver cells by inactivating essential enzymes and altering lipid metabolism (*46*). In normal conditions, most of these reactive species can be neutralized by antioxidants in the body, such as glutathione (GSH) and vitamin E. However, as discussed above (detailed in 1.2), alcohol-induced disruption in absorption,

digestion, and biosynthesis cause decreased GSH production from S-adenosylmethionine and decreased vitamin E absorption from food. As a result, the antioxidant defense system in the body is impaired, which enables the reactive species to interfere with normal cellular functions.

Overall, alcohol metabolism through the MEOS can lead to liver damage via producing toxic acetaldehyde, inducing hypoxia mediated injury, generating harmful reactive species, and impairing antioxidant defense system.

1.2.3 Aldehyde dehydrogenases (ALDH)

After ethanol is metabolized to acetaldehyde, ALDHs convert acetaldehyde to acetate. The ALDHs are distributed in various tissues, with the highest levels in the liver (*54*). Based on their kinetic properties and genetic sequences, ALDHs are classified as ALDH1 (low K_m, cytosolic), ALDH2 (low K_m, mitochondrial) and ALDH3 (high K_m, expressed in tumors, stomach and cornea) (*55*). Both cytosolic ALDH1 and mitochondrial ALDH2 are important for the oxidation of acetaldehyde (*56*). Same as ADH, aldehyde dehydrogenase (ALDH) is responsible for metabolizing the bulk of ethanol consumed as part of the diet and its activity contributes to the rate of ethanol elimination from the blood. In addition, the polymorphism of ALDH gene demonstrates the vital role of ALDH in ethanol oxidation. For example, ALDH2 deficiency in a large proportion of Asian population is associated with the flushing reaction due to the accumulation of acetaldehyde (*57, 58*). Thus, ALDH2 appears to play a crucial role in controlling the level of acetaldehyde during alcohol oxidation and determining its toxicity (*59*).

1.2.4 Acetyl-CoA synthetase (ACS)

Acetyl-CoA synthetase (ACS) catalyzes the ligation of acetate with CoA to produce acetyl-CoA. Once acetyl-CoA is generated, elemental components of alcohol can be integrated into major intermediary metabolism pathways, including TCA cycle, fatty acid synthesis and ketogenesis. According to the different intracellular locations, there are 2 types of ACS. One, designated as ACS1, is a cytosolic enzyme (*60*); the other ACS2, is a mitochondrial matrix enzyme (*61*). Although the two enzymes exhibit similar affinity for acetate, the regulation of the two enzymes is completely different. ACS1, mainly in hepatic cytosolic, provides acetyl-CoA for the synthesis of fatty acids and cholesterol (*60*). Consistent with the function of ACS1, it is regulated by sterol regulatory element-binding proteins, key transcriptional factors that mediate cholesterol and fatty acid synthesis in the liver (*62*). In contrast, ACS2 which is abundant in the heart and skeletal muscle and absent in liver can be induced under ketogenic conditions. In alcoholic subjects, because chronic malnutrition related to alcohol abuse (detailed in 1.3) reduces the flux of TCA cycle (*63*), acetyl-CoA is channelled to fatty acid synthesis and ketogenesis, contributing to alcohol-induced hepatosteatosis and ketosis (*64*).

1.3 MALNUTRITION AND ALCOHOLIC LIVER DISEASE

Besides ethanol metabolism, malnutrition is considered as another important cause of the liver injury in ALD based on the prevalence of malnutrition in alcoholics. The malnourished status in alcoholic subjects is developed either because they ingest too little of essential nutrients or because alcohol itself prevents the body from properly absorbing,

digesting, and utilizing those nutrients. Consequently, the deficiency of essential nutrients, such as proteins and vitamins, contributes to ALD and other alcohol-related disorders (Figure 1.3).

1.3.1 Decreased caloric intake

Ethanol has considerable energy value as 7.1 kcal/g. Therefore, substantial use of alcohol has profound effects on energy homeostasis of the body. Though alcohol is rich in energy, alcohol-derived calories doesn't have the same biological value as calories derived from other macronutrients and chronic consumption of alcohol or administration of high doses of alcohol does not produce the expected gain in body weight in some conditions (*65, 66*). The direct cause behind the decrease of caloric intake is lower food intake. It has been showed that people with ALD substitute calories from food with calories from alcohol and this may be partially explained by the diversion of funds from the purchase of food to that of alcohol (*67*). However, the decreased ingestion of nonalcohol calories could not explain the similar demonstration on hospitalized patients with ALD given adequate access to nutrition (*68*). It is postulated that the suppression of appetite caused by alcohol consumption is a key reason for decreased dietary intake of non-alcohol calories (*69*). Besides, depressed consciousness (*70*) during inebriation, hangover and gastroduodentitis due to ethanol partly also explains the decreased food intake (*37*). In addition to decreased food intake, another possible explanation of decreased caloric intake is that some of the energy produced by alcohol is "lost" or "wasted" when ethanol is

metabolized by the MEOS, thus not available to the body for producing or maintaining body weight (*66*).

1.3.2 Decreased absorption and digestion of nutrients

In addition to decreased caloric intake, alcohol also interferes with the intestinal functions in the absorption and digestion of nutrients, and therefore contributes to poor nutrition in patients with ALD (*68, 71, 72*).

Firstly, excessive alcohol consumption results in mechanical alterations in the gut, leading to disturbances in the intestinal absorption of nutrients. Mucosal damage in the small intestine was showed in both animal and human studies after either acute administration of alcohol or chronic alcohol consumption. Loss of mucosal epithelium at the tips of the villi and haemorrhagic erosions in the lamina propria was found in those study subjects (*73, 74*). Furthermore, a lack of structural proteins in the gut wall contributes to the intestinal swelling which constricts the capacity of the absorption of nutrients in the gut.

Secondly, ethanol can influence the function of enzymes involved in the nutrients absorption and digestion in several aspects: 1) Alcohol interferes with the activity of many enzymes located in the enterocytes. These enzymes include disaccharidases (lactase, sucrase, maltase) required for carbohydrate digestion and absorption and those involved in energy-generating process such as mitochondrial electron transport chain (*74, 75*). 2) Alcohol may impair the output of bile from the liver, resulting in decreased

absorption of lipid and lipid-soluble vitamins such as vitamin A and E (*76*). 3) Alcohol-induced pancreatitis diminishes excretive function of pancreas, resulting in the decreased output of enzymes necessary for absorption of lipids and proteins (*76*).

1.3.3 Decreased processing and storage of nutrients

The altered functional mass of the liver due to the increased deposits of fat and collagen may result in decreased stores of vitamins and carbohydrates. In the progress of alcoholic liver disease, especially at the stage of fatty liver, the metabolism of macronutrients including carbohydrates, lipids, and proteins is altered due to the preferential metabolism of alcohol in the liver (Mezey 1991). Decreased fatty acid oxidation in mitochondria and increased glycerolipid synthesis together contribute to the lipid deposition in the liver (*77*) and may propagate fibrosis by stimulating collagen formation (*68*). Due to morphological and functional change of the liver, less vitamins and carbohydrates are stored in the liver. More importantly, glycogen, which is the reserve of glucose in the body, is reduced as well. Consequently, the body needs to make use of alternative sources of energy such as the breakdown of muscle (*68*). This may explain the observed loss of body weight, increased nitrogen excretion in the stool, and negative nitrogen balance in patients with ALD (*76*). A summary of alcohol-induced physiological changes is presented in Figure 1.3.

1.3.4 Consequences of nutrients deficits in ALD

Proteins and vitamins are two classes of nutrients that are influenced by alcohol consumption. The deficiency of proteins and vitamins lead to severe outcomes. Proteins have diverse functions and are the chief actors within the cell, including enzymes responsible for almost all biochemical reactions occurring in the cells, messenger proteins that mediate signal transduction, and structural proteins that maintain the integrity of cellular infrastructure (*78*). Alcohol can disrupt the metabolic processes related to the homeostasis of proteins and amino acids, contributing to various clinical symptoms in patients with ALD (*79*). For example: 1) Decreased production of albumin disturbs the balance of osmolality, and can cause abnormal accumulation of fluid in the abdomen of patients with alcohol-induced cirrhosis (*80*). 2) Down-regulated urea production by alcohol results in the accumulation of ammonia and the disruption of pH balance in the body, which may increase the likelihood that patients develop hepatic encephalopathy (*81*, *82*). 4) Deficiency of amino acid S-adenosylmethione, the precursor for GSH, results in the incapability of anti-oxidation defense system in removal of the toxic byproducts of alcohol metabolism and the impairment of cell membrane repair system (*68*). 5) The immune system is suppressed due to alcohol altered protein metabolism, leading to decreased circulating antibodies needed to fight against infection (*83*).

Vitamins are required as a nutrient in tiny amounts by an organism to maintain a normal metabolism and function. They cannot be synthesized in sufficient quantities by human body, and must be obtained from the diet (*84*). Insufficient vitamin levels, particularly retinol (Vitamin A), thiamine, riboflavin, pyridoxine and ascorbic acid as well as folic acid in the alcoholics can lead to serious diseases. Heavy alcohol consumption reduces

vitamin A level and impairs the ability of the eye to adjust to darkness. Deficiency in vitamin E may impair its protective effect as antioxidants against alcohol-induced lipid peroxidation (*68*).

Overall, malnutrition caused by alcohol consumption, can lead to liver damage and impaired liver function. This is partially due to the deficits of key proteins, amino acids and vitamins that play important physiological roles in cells and the disturbances of macronutrient metabolism (Figure 1.3). Based on this point of view, researchers initially thought that malnutrition, rather than alcohol itself, was responsible for ALD (*85*). However, through the past decades of research on the relationship between alcohol, alcohol metabolism and nutrition, a more comprehensive theory is established, that is, nutritional effects and the toxic effects of alcohol both contribute to the pathogenesis of ALD and may interact with each other. This offers an alternative aspect for biomarker seeking as focuses on the influence of alcohol on nutritional small molecule metabolome which can indicate the *in vivo* change caused by alcohol consumption.

1.4 PRINCIPLE AND PRACTICE OF METABOLOMICS

Compared to the traditional analytical approach, which provides limited information on targeted genes, proteins and metabolites, the new development in high-throughput analytical platform significantly facilitates the study of complex biological systems and enables the creation of new scientific insights. To examine the fundamental components of biological system, which are genomes (complete genetic), transcriptomes (gene expressions), proteomes (protein expressions), and metabolome (metabolites), the

corresponding systems biology tools, which are genomics (*86*), transcriptomics (*87*), proteomics (*88*), and metabolomics (*89*), have been developed to generate a holistic point of view of complex biological system.

1.4.1 Metabolomics

Metabolomics is the scientific study of chemical processes involving metabolites. Similar to genomics, transcriptomics and proteomics, metabolomics focuses on the study of whole system, instead of individual component of the system. However, unlike the other "–omics" which only indicate the potential for physiological change, metabolomics measures real end-points of physiological processes and reflects the unique chemical fingerprints that specific cellular processes produce (*90, 91*). As each "-omics" has its advantages and limitations, an integration of genomic, transcriptomic, proteomic, and metabolomics information is expected to give a more comprehensive picture of living organisms.

1.4.2 Application of metabolomics on nutritional study

The classical analytical approach in nutrition research is generally hypothesis-driven and has specific targets. This approach has its merit in unraveling mechanisms in many nutritional events, but also has its limitation on identifying new metabolic pathways and mechanisms. Unlike the metabolism of pharmacological agents, the metabolism of nutrients involves multiple organs and targets and affects many metabolic pathways and functions. Furthermore, the influence of nutrients on biological system may not be as

apparent as pharmacological agents and usually requires longer time to demonstrate the effects. As a result, a more sensitive and multiple-targeted approach is needed. Metabolomics, as an analytical approach that allows to analyze hundreds of metabolites simultaneously in a given biological sample and detect subtle changes in a large dataset, has been broadly applied in nutritional research. For example, metabolomics has been used to characterize the effects of both a deficiency or a supplementation of different nutrients and to evaluate the metabolic effects of different foods (*92, 93*). Novel biomarkers of food intake that are predicted from current knowledge of food composition can also be identified via the means of metabolomics approach (*94*). In addition, it may also enable the separation of the effects of diet from those of confounding factors such as age, gender, physiological states and genotype and improve the understanding of the metabolic responses of humans or animals to dietary interventions (*95*).

1.4.3 Instrumentation for metabolomics

There is no single analytical method currently capable of extracting and detecting all metabolites. Every analytical platform used for metabolomics has their own pros and cons, thus researchers need to choose the appropriate analytical instrument based on the needs of the study. Most commonly-used analytical instruments in metabolomics research are nuclear magnetic resonance (NMR) spectrometers and mass spectrometry (MS). Overall, NMR has advantages such as the non-destructive nature of sample preparation and the comprehensive coverage of chemical species, while MS possesses much better sensitivity and resolution as well as high-throughput capacity (*96*).

Although NMR is widely used in many metabolomics studies, an increasing number of nutrition studies based on MS technology have been published in recent years, due to the high sensitivity and resolution and the wide availability of MS instruments (*97*, *98*). Introduction of prepared biological samples into a mass spectrometer can be through direct injection, gas chromatography (GC), liquid chromatography (LC) as well as capillary electrophoresis (*99*). Among them, LC-MS is the most widely used instrument since LC-based sample introduction results in lesser ion suppression and higher resolution than direct infusion, and it generally avoids the chemical derivatization that is generally required for GC-MS (*96*).

The initial step of LC-MS strategy is the separation of a mixture of compounds for further analysis. Traditionally, HPLC has been main staple of LC instruments. Recent development on ultra-performance liquid chromatography (UPLC) brings a much higher resolution for analyte separation and a lower limit of detection for ions (*100*) which serves to identify, quantify and purify the individual components of the mixture. After the separation, MS instruments with high-resolution mass measurement, such as time-of-flight (TOF) or Fourier transform (FT) mass spectrometers are applied to quantify with high selectivity and sensitivity and to identify metabolites. The basic principles of MS are: 1) the ionization of the molecules which leads to fragments of various mass-to-charge ratios (*m/z*), 2) the detection of the different *m/z* in the mass analyzer and 3) the identification of the compounds based on the accurate mass and the unique fragmentation pattern. Overall, high-resolution and reproducible LC-MS measurement sets up the basis for subsequent data processing and multivariate data analysis.

1.4.4 Data processing and multivariate data analysis (MDA)

Data generated from both chromatogram and spectra needs to be properly processed before introduced to MDA. General procedures include data condensation and reduction by centroiding and deisotoping mass spectra; chromatographic alignment to reduce the variation in retention time; filtering to remove noise or background signals; and peak recognition and collection by setting threshold windows for mass (*m/z*) and retention time (RT) (*101*). MS data should also be normalized to decrease the influence of systematic and sample biases, by either the parameters of the complete dataset (such as total ion count, median ion count, etc.) or the intensities of single or multiple internal standards (such as creatinine in the case of urine) (*96*). Each pair of RT-m/z is unique and acts as the identity of one compound. Such information is extracted from the chromatogram of each sample and the extracted data compiled in a table (Figure 1.4) with y rows (samples) and x columns (variables: RT-*m/z*). At this point, processed datasets can be directly used for MDA, or be further statistically transformed and scaled according to the properties of data and the purpose of MDA analysis.

Multivariate data analysis instead of other traditional statistical methods is used to handle and interpret the large data sets generated by LC-MS based metabolomics analysis (*102*), in which, a model containing one or multiple principle components (PC) can be established to represent a large portion of examined dataset. In contrast to other statistical techniques, such as t-test and ANOVA, an established MDA model and its PCs can be presented in the scores plot, in which sample-PC and sample-sample relationships can be

visualized. In LC-MS-based metabolomics, the spatial distance between two samples in the scores plot reflects their differences in chemical composition. When a clear sample clustering is observed in the scores plot, the contribution of individual ions to PCs and to the group separation can be further examined in the loadings plot, in which the relationships between ions and PCs are depicted. With appropriate MDA modeling, ions contributing to the sample separation can be detected in the loadings plot and further characterized (*96*).

There are two major categories of MDA methods, unsupervised and supervised MDA, which have been widely used in metabolomics data analysis. In unsupervised MDA, sample classification is unknown or intentionally blinded to the analytical software, while in supervised MDA this information is provided to the software for the purpose of model construction. The most popular unsupervised method is principal components analysis (PCA). Because of its indiscriminate nature, the markers identified in a robust PCA model can usually be validated. Supervised MDA encompasses many methods, including partial least squares (PLS); orthogonal partial least squares (OPLS); soft-independent modeling of class analogy (SIMCA) and partial least squares-discriminant analysis (PLS-DA) (*103*). The selection of supervised MDA method is determined by the data properties and the aim of MDA analysis.

1.4.5 Identification of novel metabolites and metabolism pathways

Combination of LC-MS and MDA has been applied and proved very efficient in identifying metabolites of either food or drugs and elucidating *in vivo* metabolic

pathways and overall metabolic maps since 2003 (*104-106*). A straightforward approach for identifying *in vivo* dietary metabolites is to compare the LC-MS chromatograms of biological samples (urine, serum or tissue extract) collected after parallel control diet intake and specific diet intake. Accurate mass-based elemental composition analysis, MS/MS fragmentation and the comparison with authentic standards allow the determination of the structures of interested metabolites. However, sometime, it is difficult to identify *in vivo* metabolites from thousands of chemical species only through visual examination of LC-MS chromatograms and fragmentation analysis when xenobiotics can also alter or disrupt the endogenous metabolic processes. Therefore, the ions contributing to the separation of control and treatment groups in the loadings plot include both xenobiotic metabolites and endogenous ions induced by the xenobiotic treatment. In this case, a stable isotope-based metabolomics approach is proposed to facilitate the identification of xenobiotic metabolites and endogenous metabolites. With appropriate data processing, the separation of vehicle-treated and xenobiotic-treated sample groups can be achieved in the scores plot of a multivariate model, and xenobiotic metabolite ions can be conveniently identified by analyzing ions contributing to the separation of xenobiotic treatment from vehicle treatment (*107*).

Overall, LC-MS-based metabolomics techniques that combine sensitivity and selectivity possess the great promise of becoming standard tools for identifying metabolites and elucidating metabolic pathways. Metabolomics approaches are superior to those traditional biochemical methods due to the clear advantages in the capacity of handling large datasets and in the graphical representation of metabolism-related sample

classification, as well as in the indiscriminative nature of metabolite identification. Although there are still limitations and unsolved problems exist, LC-MS-based metabolomics has broadened its way in nutrition research. Further effects are needed to increase the availability of LC-MS instruments and enhanced functions of MDA software in order to better serve metabolism research field.

Figure 1.1

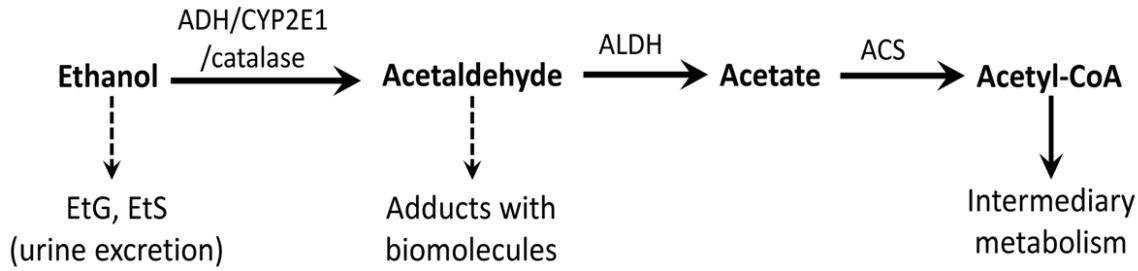

Figure 1.1 Summary of the pathways responsible for ethanol metabolism. Major metabolic pathways of ethanol (arrow with a solid line), encompassing three enzymatic steps and two intermediates, are responsible for converting ethanol into acetyl-CoA, a central metabolite of intermediary metabolism, while minor metabolic pathways (arrow with a dashed-line) affect the toxic effects of ethanol, i.e., the adductions of acetaldehyde with biomolecules contribute to the toxicity, but the formations of EtG, EtS and NAT facilitate the excretion of ethanol and acetate.

Figure 1.2

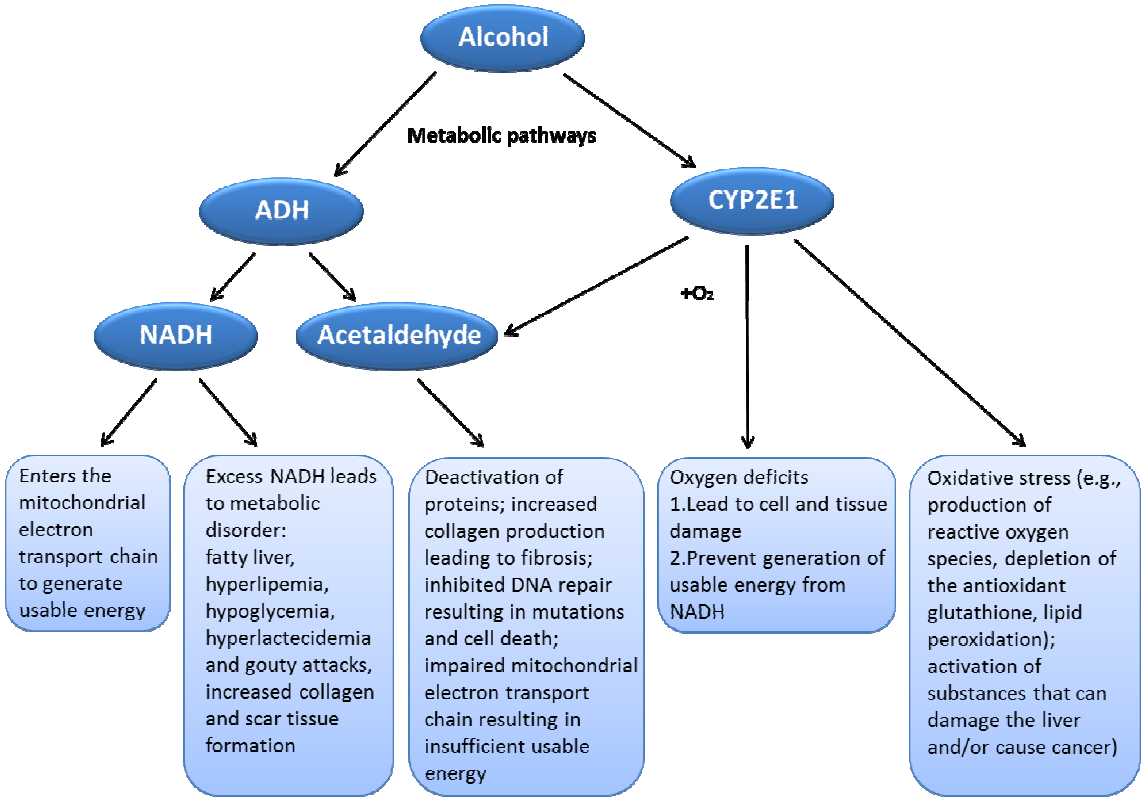

Figure 1.2 Summary of the toxic effects of chronic alcohol consumption caused by alcohol oxidation via ADH and CYP2E1 pathways. Alcohol oxidation by ADH results in formation of excess levels of NADH, which can cause various metabolic problems. Moreover, both ADH and the CYP2E1 convert alcohol to acetaldehyde, a toxic molecule that has numerous adverse effects. Alcohol also enhances the activity of CYP2E1, which generates oxidative stress in the cells, characterized by excess levels of ROS, abnormal lipid breakdown resulting in additional reactive molecules, and/or reduced levels of antioxidants which can eliminate reactive molecules. Adopted from (*46*)

Figure 1.3

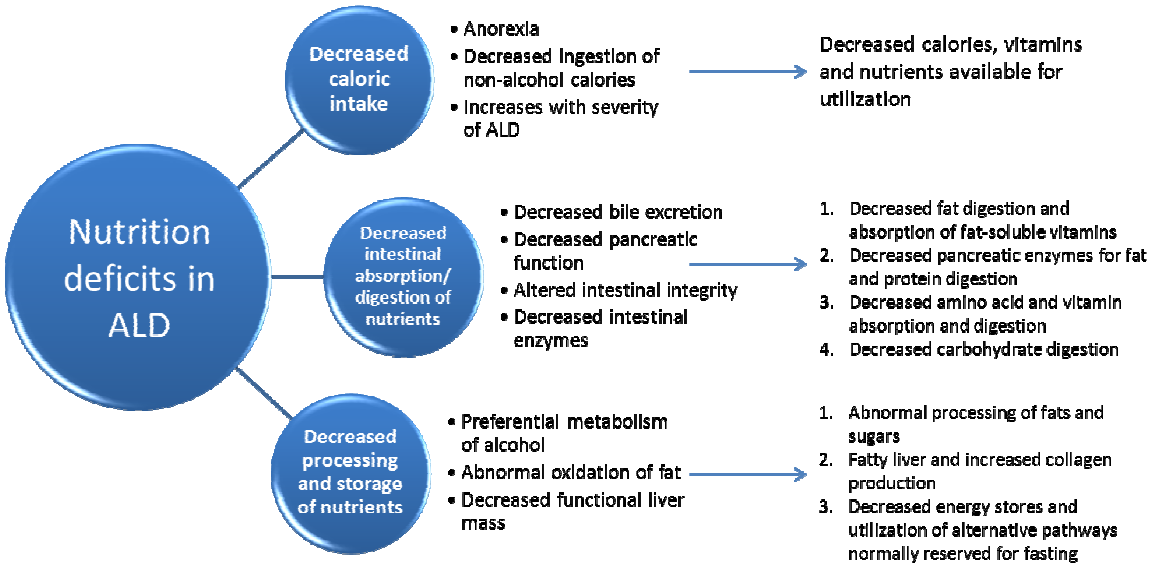

Figure 1.3 Summary of the basis for nutritional deficits in alcoholic liver disease. Adopted from (*76*)

Figure 1.4

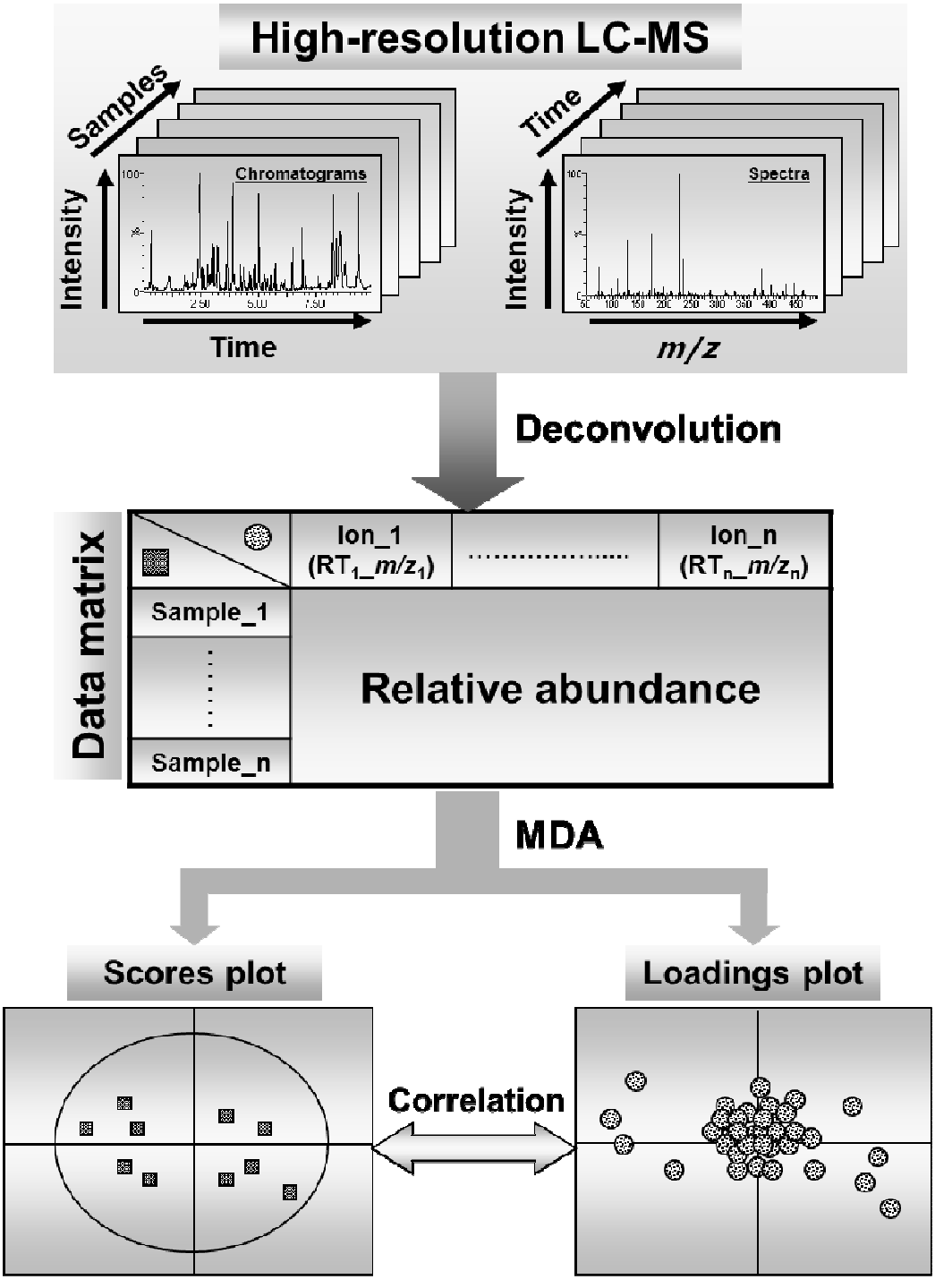
High-resolution LC-MS
Samples
Chromatograms
Intensity
Time
Time
Spectra
Intensity
m/z
Deconvolution
Data matrix
Ion_1
(RT1_m/z1)
Ion_n
(RTn_m/zn)
Sample_1
Sample_n
Relative abundance
MDA
Scores plot
Loadings plot
Correlation

Figure 1.4 The work flow of LC-MS-based metabolomic data analysis. Chromatographic and spectral data are acquired by high resolution LC-MS. Subsequent data processing, such as centroiding, deisotoping, filtering, peak recognition, yields a data matrix containing information on sample identity, ion identity (RT and *m/z*) and ion abundance. With appropriate data transformation and scaling, a multivariate model can be established through unsupervised or supervised MDA. The scores plot illustrates the principal or latent components of the model and sample classification, while the loadings plot presents the contribution of each ion to each principal component of the MDA model. Adopted from (*96*).

Chapter 2

Metabolomic Investigation of Alcohol-Induced Fatty Liver

[2] **Key Words:** Alcohol, CYP2E1, N-acetyltaurine, Acetate, Taurine, Metabolomics, Stable isotope labeling

[2] **Abbreviations**: CYP2E1, cytochrome P450 2E1; LC-MS, liquid chromatography-mass spectrometry; EtG, ethyl glucuronide; EtS, ethyl sulfate; NAT, N-acetyltaurine; ALD, alcoholic liver disease; Acetyl-CoA, acetyl-coenzyme A; CoA, coenzyme A; ADH, alcohol dehydrogenase; MEOS, microsomal ethanol-oxidizing system; ROS, reactive oxygen species; CDT, carbohydrate-deficient transferrin; MCV, mean corpuscular volume; NMR, nuclear magnetic resonance; TAG, triacylglycerol; ALT, alanine aminotransferase; BUN, blood urea nitrogen; TOF, time-of-flight; PLS-DA, partial least squares-discriminant analysis; MDA, multivariate data analysis; RT, retention time; PC, principle components; PCA, principal components analysis; PLS, partial least squares; OPLS, orthogonal partial least squares; ALDH, aldehyde dehydrogenase; ACS, acetyl-CoA synthetase; SIMCA, soft-independent modeling of class analogy;

2.1 SUMMARY

The influence of ethanol on small-molecule metabolome and the role of CYP2E1 in ethanol-induced hepatotoxicity were investigated using liquid chromatography-mass spectrometry (LC-MS) based metabolomics platform and *Cyp2e1*-null mouse model. Histological and biochemical examinations of ethanol-exposed mice indicated that the *Cyp2e1*-null mice were more resistant to ethanol-induced hepatic steatosis and transaminase leakage than the wild-type mice, suggesting CYP2E1 contributes to ethanol-induced toxicity. Metabolomic analysis of urinary metabolites revealed time- and dose-dependent changes in the chemical composition of urine. Along with ethyl glucuronide (EtG), ethyl sulfate (EtS), N-acetyltaurine (NAT) was identified as a urinary metabolite that is highly responsive to ethanol exposure and is well correlated with the presence of CYP2E1. Subsequent stable isotope labeling analysis using deuterated ethanol determined that NAT is a novel metabolite of ethanol. Among three possible substrates of NAT biosynthesis (taurine, acetyl-CoA and acetate), the level of taurine was significantly reduced while the levels of acetyl-CoA and acetate were dramatically increased after ethanol exposure. *In vitro* incubation assays suggested that acetate is the main precursor of NAT, which was further confirmed by the stable isotope labeling analysis using deuterated acetate. The incubations of tissues and cellular fractions with taurine and acetate indicated that the kidney has the highest NAT synthase activity among tested organs while the cytosol is the main site of NAT biosynthesis inside the cell. Overall, the combination of biochemical and metabolomic analysis revealed NAT is a novel metabolite of ethanol and a potential biomarker of hyperacetatemia.

2.2 INTRODUCTION

Alcohol abuse is an important health issue in the United States and around the world (*108*). Ethanol, due to its high permeability across cellular membrane, can inflict adverse effects on various parts of the body, including central nervous system, cardiovascular system, and hepato-gatrointestinal organs (*109-111*). The most common ailment associated with alcohol abuse is alcoholic liver disease (ALD), a type of hepatic dysfunction caused by ethanol-induced liver injury (*112*). Over the past decades, numerous efforts have been undertaken to understand the molecular mechanism of these complex pathophysiological events (*113-115*). However, many aspects of ethanol-induced pathogenesis remain largely unknown.

Based on currently available knowledge on ethanol-induced toxicities, the most important contributing factor in these events is the metabolism of ethanol itself (*116*). The central route of ethanol metabolism is the biotransformation of ethanol into acetyl-coenzyme A (acetyl-CoA) through a three-step reaction process, which comprises the consecutive oxidation of ethanol to acetaldehyde and then to acetate, followed by the esterification of coenzyme A (CoA). The formation of a large quantity of acetyl-CoA and reduced NADH from this process directly disrupts the nutritional and metabolic homeostasis (*37*). In addition, ethanol metabolism also produces reactive species, including acetaldehyde and free radicals, which can directly attack proteins, lipids and many other cellular components (*32*). As the consequence of ethanol-induced protein adduction (*117*), lipid peroxidation and oxidative stress (*118*), the functions of proteins and the structure of

cellular membrane are altered, leading to mitochondrial damage, pro-inflammatory responses, and impairment of antioxidant system (*119, 120*).

Enzymes responsible for initiating ethanol metabolism, which is the oxidation of ethanol to acetaldehyde, have been identified as alcohol dehydrogenases (ADH) (*121*), catalase (*122*), and cytochrome P450 2E1 (CYP2E1) (*45*). The metabolism of small amount of ethanol from intestinal flora or moderate alcohol consumption in the liver is mainly conducted by ADHs due to their relatively lower K_m value (*35, 123*), while catalase-mediated acetaldehyde production is potentially important in the extrahepatic tissues, especially in the brain (*31*). CYP2E1, a microsomal enzyme that is inducible after repeated ethanol exposure, was identified as a key component of microsomal ethanol oxidizing system (MEOS) (*124*), which is capable of generating reactive oxygen species (ROS) during the conversion of ethanol to acetaldehyde. A pathogenic role of CYP2E1 in ethanol-induced toxicities has been suggested based on its distribution, expression pattern and enzymatic property (*125*), but was not well validated due to the inconsistent results obtained from several animal studies using the *Cyp2e1*-null mice, which are deficient of CYP2E1 activity. In two recent studies, ethanol-induced hepatosteatosis and oxidative stress were significantly higher in the wild-type and the CYP2E1 humanized mice than the *Cyp2e1*-null mice, suggesting a contributing role for CYP2E1 in ethanol-induced liver injury (*126, 127*). However, in two other studies, no clear difference between the wild-type and the *Cyp2e1*-null mice was observed with regard to the adverse effects of ethanol treatment (*128, 129*). Therefore, additional studies are needed to determine whether CYP2E1 can significantly contribute to the ethanol-induced liver injury.

One prominent feature of ethanol-induced chronic toxicities is the gradualism of disease development, i.e. ALD always starts from a reversible stage of hepatosteatosis, and then gradually progresses to the irreversible stages of hepatic fibrosis and cirrhosis, and liver cancer in certain cases (*9*). As the modulations of biochemical pathways and metabolic reactions occur at each stage of ethanol-elicited diseases, examining these events in cellular and molecular levels provides an excellent venue for discovering specific and sensitive biomarkers that can indicate the extent of ethanol abuse and the scope of tissue damage. Many analytical approaches have been adopted for this purpose. For instance, metabolite analyses have identified EtG and EtS, two minor metabolites of ethanol, as the indicators of ethanol exposure (*28, 29*), while enzymatic assays have established γ-glutamyltransferase and transaminases as the markers of liver injury (*23, 24*). In addition, carbohydrate-deficient transferrin (CDT) in the serum, mean corpuscular volume (MCV) of red blood cell, and acetaldehyde adducts, have also been used for detecting ethanol abuse (*23, 25, 26*). Recent arrival of metabolomics, a global system biology methodology for measuring small-molecule metabolite profiles and fluxes in biological matrices (*96*), offers a new and powerful tool to characterize the metabolic changes induced by ethanol and to identify the small-molecule biomarkers of ethanol-induced toxicities. Mass spectrometry (MS)-based and nuclear magnetic resonance (NMR)-based metabolomic analyses of urine, blood and tissue samples have revealed the changes of organic acids (*130*), amino acids (*131*) and their derivatives (*132*), as well as fatty acids and associated lipid species (*133*) in the biofluids following ethanol treatment.

In this study, the metabolic events elicited by ethanol exposure and the role of CYP2E1 in ethanol-induced hepatotoxicity were investigated using LC-MS-based metabolomics as the analytical platform and the *Cyp2e1*-null mice as the animal model. NAT, a urinary metabolite that is formed mainly by the enzymatic reaction between acetate and taurine, was identified as a novel metabolite of ethanol *in vivo*.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

Ethanol, [$^2H_6$]-ethanol, [$^2H_4$]-acetate, and creatinine were purchased from Sigma-Aldrich (St. Louis, MO). N-acetylneuraminic acid, tri-phenylphosphine, and 2-picolylamine were purchased from Alfa Aesar (Ward Hill, MA). Dehydrated agar, 4-hydroxyphenylacetic acid, p-chlorol-L-phenylalanine, taurine and dansyl chloride LC-MS-grade water, acetonitrile, formic acid and acetate were purchased from Fisher Scientific (Houston, TX). EtG was purchased from Toronto Research Chemical (North York, Ontario, Canada), acetyl-CoA from Roche (Branford, CT) and 2,2'-dipyridyl disulfide from MP Biomedicals (Solon, OH).

2.3.2 Animals and ethanol treatments

Male wild-type and *Cyp2e1*-null mice on the 129/Sv strain background (*159*), 10-12-week old, were used in this study. All animals were maintained in a University of Minnesota (UMN) animal facility under a standard 12 h light/12 h dark cycle with food and water ad libitum. Handling and treatment procedures were in accordance with animal study protocols approved by the UMN Animal Care and Use Committee. A modified semi-solid diet that is formulated based on Lieber-DeCarli liquid ethanol diet was supplied to mice using customized feeding tubes (*160, 161*) (Figure 2.1). To be specific, 0.5% (w/w) agar powder was dissolved in warm water (1/2 of the total water volume). The control dextrose diet powder and the Lieber-DeCarli ethanol diet powder (Bio-Serv)

were mixed in a ratio based on desired ethanol concentration and then suspended in water (the other 1/2 of the total water volume). Two solutions were blended together and ethanol was added to reach to desired concentration. Prepared semi-solid ethanol diet was stored in air-tight container at 4°C and is used within a week. To acclimate to the semi-solid diet, both wild-type and *Cyp2e1*-null mice were initially fed the control semi-solid dextrose diet for 3 days before the treatment. Afterward, the mice were fed either the ethanol diet or the control dextrose diet for 21 days. The concentration of ethanol was increased weekly from 2.2% (v/v) to 4.5% (v/v) and finally 5.4% (v/v) (Figure 2.2A). The amount of food consumption of each group was monitored and was determined as statistically comparable.

2.3.3 Sample collection and liver histology

Serum samples were collected by submandibular bleeding. Urine samples were collected by housing mice in metabolic cages for 24 hours. The liver and other tissue samples were harvested after mice were euthanized by carbon dioxide. All tissue samples were stored at -80°C before further analysis except one aliquot of liver tissue that was immediately fixed in 10% formalin solution after dissection, and then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for general histology.

2.3.4 Preparation of tissue homogenates and intracellular fractions

Tissue homogenates from liver, kidney, heart, muscle and brain were prepared by the homogenization in a buffer containing 320 mM sucrose, 50 mM phosphate buffered

saline (PBS) solution, 1 mM EDTA and protease inhibitor and then the centrifugation at 600 × g for 10 min to remove nuclear pellet. Intracellular fractions were obtained by stepwise centrifugation. Briefly, the tissue homogenates of liver and kidney were centrifuged at 9000 × g for 20 minutes. Mitochondrial fraction was prepared by washing, centrifuging and then reconstituting the pellet in a suspension buffer containing 100 mM PBS, 20% (v/v) glycerol, 1 mM EDTA and protease inhibitor. The supernatant from 9000 × g centrifugation was further centrifuged at 100,000 × g for 1 hour. The resulting supernatant was the cytosolic fraction while the precipitate was reconstituted in the suspension buffer as the microsomal fraction.

2.3.5 Biochemical assays

Serum alanine aminotransferase (ALT) activity, liver and serum triacylglycerol (TAG) level, and blood urea nitrogen (BUN) level were measured using the colorimetric assay kits from Pointe Scientific (Canton, MI). The lipid fraction of the liver was prepared by chloroform-methanol extraction (*162*).

2.3.6 Western blotting

The expression level of CYP2E1 in the mouse liver microsome was measured using a monoclonal antibody (1-98-1) against mouse CYP2E1 (*163*). Calnexin was used as the loading control of microsomal proteins.

2.3.7 Urine sample preparation and LC-MS analysis

Urine samples were prepared by mixing 50 µL of urine with 200 µL of 50% aqueous acetonitrile and centrifuging at 21,000 × g for 10 min to remove protein and particulates. Supernatants were injected into a Waters Acquity™ UPLC system (Milford, MA) and separated by a gradient of mobile phase ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10-min run. LC eluant was introduced into a Waters SYNAPT QTOF mass spectrometer (QTOF-MS) for accurate mass measurement and MS/MS analysis. Capillary voltage and cone voltage for electrospray ionization (ESI) was maintained at 3 kV and 30 V for positive-mode detection, and at -3 kV and -35 V for negative mode detection, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range m/z 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephpalin ($[M+H]^+$ = 556.2771 m/z and $[M-H]^-$ = 554.2615 m/z) in real time. After data acquisition in QTOFMS, chromatograms and spectra of urine samples were processed by MassLynx™ software (Waters).

2.3.8 Chemometric analysis and biomarker identification

Chromatographic and spectral data of urine samples from the wild-type and *Cyp2e1*-null mice were deconvoluted by MarkerLynx™ software (Waters). A multivariate data matrix containing information on sample identity, ion identity (RT and m/z) and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition and

integration. The intensity of each ion was calculated by normalizing the single ion counts (SIC) versus the total ion counts (TIC) in the whole chromatogram. The data matrix was further exported into SIMCA-P+^{TM} software (Umetrics, Kinnelon, NJ), and transformed by mean-centering and Pareto scaling, a technique that increases the importance of low abundance ions without significant amplification of noise. Both unsupervised and supervised multivariate data analyses (MDA), including principal components analysis (PCA), projection to latent structures-discriminant analysis (PLS-DA), and orthogonal projection to latent structures (OPLS) analysis, were applied to classify the measured urine samples (*164*). Principal components were generated by MDA to represent the major latent variables in the data matrix and were described in a scores scatter plot. Potential urinary biomarkers were identified by analyzing ions contributing to the principal components and to the separation of sample groups in the loadings scatter plot.

2.3.9 Structural elucidation and synthesis of NAT

The structures of urinary biomarkers identified by metabolomic analysis were determined by accurate mass-based elemental composition analysis, MS/MS fragmentation, and the comparisons with authentic standards. The MS/MS fragmentation was conducted by using collision energy ramping from 10 to 40 eV in SYNAPT QTOF-MS.

NAT was synthesized based on a method described by Johnson et al. (*138*). Briefly, 0.5 g of taurine was dissolved in a mixture of 7 mL of water and 2.5 mL of pyridine, and then 2 mL of acetic anhydride was added dropwise. The reaction mixture was stirred at 4°C overnight. After the solvent was removed under vacuum, the product was recrystallized

using a mixture of ethanol and dichloromethane (1:1), and dried under vacuum for 10 hours. A yield of 286 mg of NAT (43%) was obtained, m.p. 99-101°C.

2.3.10 Stable isotope labeling analysis of ethanol metabolism and the biosynthesis of NAT

Semi-solid diets containing 2.2% unlabeled ethanol, 2.2% [$^2H_6$]-ethanol, or 1.1% [$^2H_4$]-acetate were fed to the wild-type mice for 7 days. 24-hour urine samples were collected and then analyzed by UPLC-QTOF-MS. Data from [$^2H_6$]-ethanol or [$^2H_4$]-acetate treatment were compared with those from unlabeled ethanol treatment through a OPLS-based metabolomic analysis (*165*). Deuterated metabolites of ethanol and acetate were identified in the S-plots of OPLS models, and further confirmed by comparing extracted ion chromatograms of unlabeled metabolites with those of deuterated metabolite and by MS/MS fragmentation.

2.3.11 Quantitation of urinary biomarkers

Urinary biomarkers were quantified by accurate mass-based ion extraction chromatograms. Standard curves of creatinine, EtG and NAT ranging from 10 μM to 500 μM were prepared using dynamic range enhancement function of SYNAPT QTOF-MS (*29*). Urinary concentrations of EtG and NAT were determined by the integration of peak area and fitting with the standard curve using QuanLynx^{TM} software (Waters) and expressed as the molar ratio to creatinine.

2.3.12 Quantitation of the precursors of NAT in the liver and serum

To determine the effects on ethanol overdose on the levels of potential precursor of NAT *in vivo*, a 4 g/kg dose of ethanol was administered to both wild-type and *Cyp2e1*-null mice by intraperitoneal (i.p.) injection (*166*). Serum samples were collected prior to the injection and at 0.5, 1, 2, 4, 6 hours after the injection. Liver samples were harvested prior to the injection and at 1 and 6 hours after the injection. To measure the amount of taurine in the liver, the sample mixture was prepared by mixing 5 µl of liver homogenate with 5 µl of 100 µM p-chlorol-L-phenylalanine (internal standard) and then followed by the addition of 40 µl of 10 mM Na_2CO_3 (pH=11). The sample mixture then reacted with 100 µl of freshly prepared dansyl chloride in acetone (3 mg/ml) by vortexing and heating at 60°C for 10 minutes (*167*). The reaction mixture was centrifuged at 21,000 × g for 10 minutes at 4°C. The supernatant was transferred to a HPLC vial and injected into the LC-MS system for quantitation. The peak area of dansyl-taurine derivative ($[M+H]^+$ = 395.0730) was monitored for determining the concentration of taurine. The standard curve ranging from 50 µM to 1000 µM was prepared using the same procedure. To measure the hepatic content of acetyl-CoA, 100 µl of liver homogenate was spiked with sulfadimethoxine (internal standard) and extracted twice by 250 µl of 50% aqueous acetonitrile. The extract was dried by vacuum and reconstituted by 50 µl of phosphate buffered saline containing 20% acetonitrile. Acetyl-CoA ($[M+H]^+$ = 810.1336) concentration was determined by LC-MS with a standard curve from 5 µM to 200 µM. To measure the amount of acetate in the liver and serum, 10 µL of liver homogenate and serum sample were diluted by 90 µL of acetonitrile, and then mixed with 10 µL of 100 µM [$^2H_4$]-acetate (internal standard). The derivatization reagents, including 10 µl of 10

mM triphenylphosphine, 10 µl of 10 mM 2,2'-dipyridyl disulfide, and 10 µl of 10 mM 2-picolylamine (PA), were added into the sample mixture consecutively (*168*). The reaction mix was vortexed and then heated at 60°C for 15 minutes. After centrifuging at 21,000 × g for 10 minutes, the supernatant was transferred to a HPLC vial and injected into the LC/MS system for analysis. The peak area of PA-acetate derivative ($[M+H]^+$ = 151.0866) was monitored for determining the concentration of acetate. The standard curve ranging from 50 µM to 1000 µM was prepared using the same procedure.

2.3.13 Enzymatic kinetics of NAT biosynthesis reactions

In vitro enzyme kinetics experiment was conducted by incubating liver homogenate, 20 mM taurine, and acetyl-CoA or acetate ranging from 100 µM to 6 mM in a phosphate buffered saline solution at 37°C for 10 minutes. Reaction was terminated by adding the equal volume of acetonitrile. NAT as the reaction product was further analyzed by LC-MS. K_m and V_{max} of the enzymes utilizing acetyl-CoA or acetate as the substrate were calculated based on Michaelis-Menten equation.

2.3.14 Sites of NAT biosynthesis

To determine the activity of NAT biosynthesis in the metabolically active organs, tissue homogenates of liver, kidney, heart, brain and muscle were incubated with 20 mM taurine and 2.5 mM acetate for 30 minutes, and the yield of NAT was measured. Similarly, the intracellular location of NAT biosynthesis was determined by incubating intracellular fractions of liver and kidney, including cytosol, mitochondria and

microsome, with 20 mM taurine and 2.5 mM acetate, followed by the quantitation of NAT.

2.3.15 Statistics

Experimental values are expressed as mean ± standard deviation (SD). Statistical analysis was performed with two-tailed Student's t-tests for unpaired data, with a P value of <0.05 was considered as statistically significant.

2.4 RESULTS

2.4.1 Different responses to ethanol feeding from the wild-type and Cyp2e1-null mice

To define the role of CYP2E1 in ethanol-induced hepatotoxicity, the wild-type and *Cyp2e1*-null mice were fed with control or ethanol diet for 21 days (Figure 2.2A). To avoid the urine and fetal contamination associated with liquid ethanol diet, a modified semi-solid ethanol diet was formulated (detailed in *Materials and Methods*) and was well received by the mice used in this study. After 21-day of ethanol feeding, CYP2E1 protein was dramatically increased in the wild-type mice while it remained absent in the *Cyp2e1*-null mice (Figure 2.2B), confirming that CYP2E1 expression is responsive to ethanol treatment (25). No significant difference in the change of body weight was observed between two mouse lines fed with the control diet. However, the body weight of wild-type and *Cyp2e1*-null mice fed with ethanol diet was slightly decreased after starting the 5.4% ethanol diet at day 15 (data not shown). Since hepatic steatosis is a well-established acute response to ethanol treatment, microscopic examination of liver histology was conducted. The results showed that the development of both microvesicular and macrovesicular steatosis around the central vein was delayed in the *Cyp2e1*-null mice compared to the wild-type mice (Figure 2.2C). Consistent to the histological analysis, the triacylglycerol (TAG) level in the liver and serum of wild-type mice was increased in a time- and dose-dependent pattern while its level in the *Cyp2e1*-null mice was not significantly altered by ethanol exposure (Figure 2.3A-B). It is noteworthy that the basal level of TAG in the liver of *Cyp2e1*-null mice was significantly lower than its level in the

wild-type mice (Figure 2.3A). This difference between two mouse lines has also been observed in a separate study (24), but its cause remains to be determined.

The ethanol-elicited liver injury was evaluated by measuring the activity of serum alanine aminotransferase (ALT). The increase of ALT activity occurred in both mouse lines after 21-day ethanol feeding, but the increase in the *Cyp2e1*-null mice was significantly less than that in the wild-type mice (Figure 2.3C). Furthermore, the effect of ethanol treatment on the kidney function of wild-type and *Cyp2e1*-null mice was determined by measuring blood urea nitrogen (BUN) level. During the 21-day ethanol feeding, the decrease of BUN level in the wild-type mice occurred after 14-day treatment, but didn't happen in the *Cyp2e1*-null mice until day 21 of the treatment (Figure 2.3D). Overall, both histological and biochemical analysis of the responses to ethanol feeding from the wild-type and *Cyp2e1*-null mice indicated that, deficiency of CYP2E1, a protein that is involved in ethanol metabolism and also inducible by ethanol exposure, reduces the toxic effects of ethanol, suggesting a contributing role of CYP2E1 in the ethanol-induced toxicity.

2.4.2 Metabolomic investigation of ethanol-induced metabolic events in the wild-type and Cyp2e1-null mice

To further explore the mechanism underlying general toxic effects of ethanol and different responses of the wild-type and *Cyp2e1*-null mice to ethanol challenge, metabolic events induced by ethanol treatment were examined through LC-MS-based metabolomic analysis of urine samples from the two mouse lines. After the data acquired

from the chromatograms and mass spectra of LC-MS analysis were processed by the projection to latent structures-discriminant analysis (PLS-DA), a type of supervised multivariate data analysis (MDA), a two-component multivariate model, represented by a scores scatter plot, was constructed to illustrate the relationship among sample groups (Figure 2.4A-B). The distinctive separation of urine samples collected at the different time points of ethanol feeding in the wild-type mice (circled and labeled in Figure 2.4B) suggested that ethanol treatment dramatically altered the chemical composition of urinary metabolome in a time- and dose-dependent pattern, implying the possibility of identifying the urinary metabolites that can function as the biomarkers of ethanol exposure. Similarly, the ethanol-exposed *Cyp2e1*-null mice were also clearly separated from their untreated controls in a PLS-DA model, even though the genotype-dependent differences between the wild-type and *Cyp2e1*-null mice, represented by the separation of two mouse lines along the principal component 2 of the model, preexist prior to the ethanol treatment (Figure 2.4A). The urinary ions contributing to the classification of sample groups were further characterized in a loadings scatter plot (Figure 2.4C) and the chemical identities of ions that are highly induced by ethanol treatment were determined by accurate mass measurement, elemental composition analysis, MS/MS fragmentation, and comparisons with authentic standards if available (Table 1). Among them (I-X), EtG (III) and EtS (IV) are the minor metabolites of ethanol (*28, 29*); 4-hydroxyphenylacetic acid sulfate (II) as well as its in-source fragment (IX) has been shown as a metabolite of intestinal flora that is highly responsive to ethanol treatment (*130*); and N-acetylneuraminic acid (X), a functional component of glycoproteins and glycolipids, has been identified as a

biomarker of alcohol abuse in the saliva and serum (*134*). The observation of these known markers of ethanol exposure indicated that the ethanol feeding method in this study was effective in altering the urinary metabolome and the multivariate analysis was efficient in identifying small-molecule markers. More importantly, NAT (I) was identified as a novel marker that is highly responsive to ethanol treatment based on its high value in the loadings scatter plot. Its chemical identity was confirmed by the comparisons of its chromatographic peak and MS/MS spectra with synthesized standard (Figure 2.4D-E).

2.4.3 Quantitation of urinary biomarkers of ethanol exposure

To validate the observation of metabolomics analysis (Figure 2.4), the levels of urinary NAT (I) and EtG (III) in the wild-type and *Cyp2e1*-null mice during 21-day ethanol treatment were compared through two different normalization approaches. One is to calculate the molar ratio to creatinine, which is commonly used to quantify urinary metabolites (*135*), the other is based on the relative abundance of individual ion in the total MS signals, which was used to construct our multivariate model on urine samples (detailed in the Materials and Methods). The result from creatinine-based normalization showed that comparable amounts of NAT existed in the urine of both wild-type and *Cyp2e1*-null mice prior to ethanol exposure, and its level remained stable in the control group, suggesting NAT is already a constitutive component of mouse urine even without ethanol treatment (Figure 2.5A). After 7-day exposure of 2.2% ethanol, the amount of NAT increased in both mouse lines to a comparable level. However, subsequent

treatments of 7-day 4.5% ethanol and 7-day 5.4% ethanol led to significantly higher levels of NAT in the wild-type mice than its level in the *Cyp2e1*-null mice (Figure 2.5A). In contrast, urinary EtG was undetectable prior to the ethanol treatment, confirming that EtG is not an endogenous compound, but an ethanol metabolite (27). As the biosynthesis of EtG doesn't require the involvement of CYP2E1, no significant difference between the ethanol-treated wild-type and *Cyp2e1*-null mice was observed (Figure 2.5B). Compared to the result from creatinine-based normalization, the result from total MS signals-based normalization showed even clearer difference between the two mouse lines on the urinary level of NAT at all three time points after ethanol treatment, including the day 7 of ethanol exposure, while no difference on EtG (Figure 2.5C-D). The discrepancy between the results from these two analytical approaches is caused by the ethanol-induced change in creatinine excretion, which was observed in our study (data not shown) and has also been reported in previous alcohol-related studies (*136, 137*). Taken together, the quantitation of urinary NAT and EtG confirmed their identities as the biomarkers of ethanol exposure because both of them were dramatically induced by ethanol. The main difference between them is that NAT, an endogenous compound, can also reflect the different responses of the two mouse lines to ethanol treatment.

2.4.4 Identification of NAT as a metabolite of ethanol through stable isotope labeling analysis

NAT has been identified as a chemical component of mouse urine in a recent metabolomics study on the radiation-induced toxicity (*138*). However, its relation with

ethanol has not been shown previously. To examine the source of newly synthesized NAT after ethanol exposure, deuterated ethanol ([$^2H_6$]-ethanol) was fed to the wild-type mice for 7 days, and the chemical composition of urine samples from both unlabeled and labeled ethanol treatments were compared through LC-MS-based metabolomic analysis. As shown in a loadings S-plot of detected urinary ions from an OPLS analysis, NAT and its isotopic counterpart, [acetyl-$^2H_3$]NAT, were identified as the most prominent ion corresponding to the unlabeled and labeled ethanol treatment, respectively (Figure 2.6A). The migration of the deuterium atoms from labeled ethanol into the acetyl group of NAT was further confirmed by the MS/MS fragmentation of [acetyl-$^2H_3$]NAT in the urine, indicating ethanol can be the metabolic precursor of NAT (Figure 2.6B). To determine the direct contribution of exogenous ethanol to NAT biosynthesis, the relative abundances of unlabeled NAT and [acetyl-$^2H_3$]NAT were further compared. The result showed that the level of unlabeled NAT, which was not directly originated from deuterated ethanol, increased slightly after ethanol exposure, but more importantly, the level of [acetyl-$^2H_3$]NAT in urine, which was from deuterated ethanol, increased dramatically, suggesting that ethanol is the major source of newly synthesized NAT after ethanol exposure (Figure 2.6C). Furthermore, the amount of ethanol that was converted *in vivo* into the urinary NAT was calculated as the percentage of daily ethanol intake through an equal-molar conversion (1 molar NAT is equivalent to 1 molar ethanol) and was compared with that of EtG. The results showed that the urinary contents of NAT and EtG are only equal to a small amount of ethanol consumed by the mice (Figure 2.6D), indicating that NAT, similar to EtG, is a minor metabolite of ethanol. Overall, the stable

isotope labeling analysis defined NAT as both an endogenous metabolite that is constitutively synthesized, and an exogenous metabolite formed by ethanol metabolism.

2.4.5 Identification of NAT as the product of the enzymatic reaction between taurine and acetate

As a taurine ester, NAT is likely formed by the esterification reaction with taurine. Since stable isotope labeling analysis revealed that ethanol can donate an acetyl group for NAT biosynthesis (Figure 2.6B), acetyl-CoA and acetate, two ethanol metabolites containing acetyl groups, were considered possible candidates of acetyl donors. To test this hypothesis, the levels of taurine, acetyl-CoA and acetate in the wild-type and *Cyp2e1*-null mice were measured after administering a bolus dose of ethanol (i.p. injection of 4 g/kg ethanol). As taurine and acetyl-CoA are highly enriched inside the cells due to their specific transport and biosynthesis mechanisms, the influences of ethanol were evaluated by measuring their levels in the liver. In contrast, acetate is highly diffusive and much more evenly distributed in the body. Hence, the acetate levels in both liver and serum were examined to determine the impact of ethanol on its turnover. Results from quantitative LC-MS analysis showed that all three metabolites were affected by ethanol treatment (Figure 2.7A-D). Among them, hepatic taurine level was significantly reduced in both wild-type and *Cyp2e1*-null mice (Figure 2.7A), suggesting that the conversion of taurine occurred quickly after ethanol challenge. In contrast to the decrease of hepatic taurine level, the concentrations of acetyl-CoA and acetate in the liver of both mouse lines were significantly increased by ethanol challenge (Figure 2.7B-C), as the

metabolism of excessive ethanol is expected to elevate their levels in the liver (*139*, *140*). One clear difference between hepatic acetate and acetyl-CoA is that the concentration of hepatic acetate was over 200-fold higher than that of hepatic acetyl-CoA. In addition, the dramatic increase of serum acetate level was observed in both wild-type and *Cyp2e1*-null mice following the ethanol challenge (Figure 2.7D). The high level of serum acetate was sustained in the wild-type mice for 6 h, but was gradually reduced in the *Cyp2e1*-null mice after 4 h of ethanol treatment (Figure 2.7D). Overall, the changes of three metabolites after ethanol challenge, i.e., the decrease of taurine and the increase of acetyl-CoA and acetate, were consistent to the increase of NAT, suggesting these changes potentially contribute to ethanol-induced NAT biosynthesis.

Since acetyl-CoA is the direct downstream metabolite of acetate in ethanol metabolism, two possible routes of NAT biosynthesis from acetate exist. One is the direct reaction of acetate with taurine, while the other is the reaction of acetyl-CoA with taurine after acetate is converted to acetyl-CoA. To examine these two routes of NAT biosynthesis, *in vitro* incubations of liver homogenate of the wild-type mice with taurine and various concentrations of acetate or acetyl-CoA were performed, and the kinetics parameters of enzyme reactions were further determined. The 20 mM taurine concentration was chosen based on the concentration of hepatic taurine in the wild-type mice (Figure 2.7A) and the reported physiological concentration of taurine (*141*). The results showed that both acetyl-CoA and acetate can react with taurine to produce NAT *in vitro* with different kinetic features of enzyme reaction (Figure 2.7E). The K_m and V_{max} values for the reaction of acetyl-CoA and taurine are 1.96 mM and 0.10 μmole/g liver/min, respectively,

while the K_m and V_{max} values for the reaction of acetate and taurine are 2.10 mM and 0.27 μmole/g liver/min, respectively. Considering the correlation between the K_m value of *in vitro* reactions (Figure 2.7E) and the metabolite concentrations in the liver (Figure 2.7B-C), it is evident that the direct reaction of acetate and taurine, instead of the reaction of acetyl-CoA and taurine, should be the main route of NAT biosynthesis *in vivo* (detailed in the Discussion).

To further determine the role of acetate in NAT biosynthesis, a stable isotope labeling analysis was conducted by feeding the wild-type mice with the diet containing 1.1% deuterated acetate ([$^2H_4$]-acetate) for 7 days. The results from monitoring both unlabeled NAT and [acetyl-$^2H_3$]NAT in the urine clearly demonstrated that the exposure of [$^2H_4$]-acetate not only quickly led to the biosynthesis of [acetyl-$^2H_3$]NAT, but also maintained its level during the 7-day treatment (Figure 2.7F), confirming that the excessive acetate is the source of newly synthesized NAT after ethanol overdose.

2.4.6 Sites of NAT biosynthesis

Although the liver is the major site of ethanol metabolism, the high level of acetate in serum makes acetate available for further metabolism in extrahepatic tissues after ethanol exposure. To determine the tissues/organs that are capable of NAT biosynthesis, the *in vitro* incubations of taurine and acetate were conducted using the homogenates of tissues/organs that are metabolically active. The result clearly showed that the kidney has the highest enzymatic activity of NAT biosynthesis from taurine and acetate, even much higher than the activity in the liver (Figure 2.8A). The contributions of heart, muscle and

brain to NAT biosynthesis *in vivo* are likely minimal based on their low activities even though their taurine contents are reportedly high (*141*). Furthermore, the intracellular locations of NAT biosynthesis were examined using subcellular fractions of kidney and liver, including mitochondria, cytosol and microsome. It is clear from the results of *in vitro* incubations that the cytosolic fractions have much higher activity of NAT biosynthesis than the mitochondrial and microsomal fractions (Figure 2.8B). In summary, *in vitro* study of NAT biosynthesis suggested that the cytosolic enzymes in kidney and liver are mainly responsible for NAT biosynthesis *in vivo*.

2.5 DISCUSSION

Ethanol metabolism has been studied extensively due to the ubiquitous presence of ethanol in nature and its association with hepatotoxicity and neurotoxicity in humans. A three-step route of ethanol metabolism has been established (*116*), in which acetaldehyde and acetate are the oxidized intermediates in the metabolic process of converting ethanol to acetyl-CoA, a central metabolite in the intermediary metabolism (Figure 2.9). Besides these major metabolic reactions, minor metabolic pathways of ethanol and acetaldehyde have also been identified and characterized as important factors in ethanol-related toxicities. Glucuronidation and sulfation, two conjugation reactions, function as a minor detoxification mechanism to convert ethanol to EtG and EtS, two unreactive metabolites that have been used as the biomarkers of ethanol consumption (*28, 29*). In contrast to the detoxification feature of ethanol conjugation reactions, non-enzymatic adduction reactions between acetaldehyde and biomolecules (proteins, DNAs) have been suggested as a major contributing factor in ethanol-induced toxicities (*142*). In this study, the formation of NAT was identified and characterized as a novel metabolic pathway of acetate, which may serve as a protective mechanism by removing excessive acetate from blood to urine (Figure 2.9).

NAT, a highly water-soluble and hygroscopic compound, was previously found in nature as a major component in the sticky droplet of orb spider web (*143*). Its presence in the biofluids of mammals has not been described until a recent study on the urine metabolome of radiation-exposed rat, in which the urinary NAT level was increased by γ-

irradiation (*138*). Our current study, for the first time, showed that NAT is a constitutive component of mouse urine and its level is dramatically increased after ethanol exposure. Stable isotope labeling analysis using deuterated ethanol further revealed that NAT is indeed a novel metabolite of ethanol (Figure 2.6C). As a taurine ester, NAT is likely formed by one or multiple N-acetylation reactions between taurine and ethanol metabolites. To test this hypothesis, the possibilities of acetyl-CoA and acetate, two ethanol metabolites containing acetyl group, as the donor of acetyl group of NAT were investigated both *in vitro* and *in vivo*. Hyperacetatemia, instead of high level of acetyl-CoA, was defined as the major contributor of NAT induction after ethanol exposure based on several facts: 1). The quantitation data showed that the concentration of acetate is much higher than that of acetyl-CoA *in vivo* both before and after ethanol exposure (Figure 2.7B-C). Hence, the concentrations of acetate in the liver and serum are comparable to the determined K_m value of the reaction between acetate and taurine, but the concentration of hepatic acetyl-CoA is far below the K_m value of the reaction between acetyl-CoA and taurine (Figure 2.7E), suggesting that the reaction between acetate and taurine is much more likely to happen *in vivo*. 2). Incubations of the cellular fractions of liver and kidney indicated that the intracellular site of NAT biosynthesis is the cytosol, not the mitochondria or microsome (Figure 2.8B). Because acetyl-CoA concentration in the cytosol is much lower than that in the mitochondria (*144*), it is unlikely that active NAT biosynthesis from acetyl-CoA occurs in the cytosol. Furthermore, this proposed NAT biosynthesis pathway was supported by observing the decrease of taurine content in the liver after ethanol challenge (Figure 2.7A) and detecting the formation of deuterated

NAT after the treatment of deuterated acetate (Figure 2.7F), suggesting the consumption of taurine and acetate for the formation of NAT *in vivo*. The enzymatic feature of NAT biosynthesis from acetate and taurine was further investigated and can summarized as follows: 1). The reaction between acetate and taurine is enzymatic since boiled liver and kidney homogenates fail to generate NAT from acetate and taurine, but can yield small amount of NAT from acetyl-CoA and taurine (data not shown). 2). The reaction between acetate and taurine is likely a one-step direct reaction which doesn't require the conversion of acetate to acetyl-CoA before reacting with taurine. This conclusion is largely based on the fact that ATP and coenzyme A (CoA), two essential cofactors in acetyl-CoA synthetase (ACS)-mediated acetyl-CoA synthesis from acetate (*145*), are not required for the NAT biosynthesis from acetate and taurine. 3). The enzyme responsible for NAT synthesis mainly locates in the cytosol of kidney and liver, as suggested by the NAT synthesis through *in vitro* incubation of various organ homogenates and intracellular fractions (Figure 2.8). In summary, the enzyme responsible for the biosynthesis of NAT (we would like to name it as "NAT synthase") is likely a cytosolic enzyme in the kidney and liver that can directly catalyze the esterification reaction between taurine and acetate without the involvement of ATP and CoA. At present, the protein identity of NAT synthase and its relation with ethanol treatment are unknown and will require further biochemical studies.

Acetate and taurine, as the sources of NAT biosynthesis, are two biochemically important metabolites. Therefore, the formation of NAT is expected to affect the metabolic pathways related to acetate and taurine, as evidenced by the increased acetate level and

the decreased taurine level after ethanol treatment in this study (Figure 2.7A-D). Acetate in the body is originated from both endogenous and exogenous sources, including the hydrolysis of acetyl-CoA, the metabolism of gut flora, and ethanol metabolism (*146*). When these acetate-producing routes are activated, such as in the diabetic humans and animals (*147, 148*) and after excessive ethanol consumption, hyperacetatemia, the high level of acetate in blood, can be readily induced. In addition, hyperacetatemia also commonly occurs in long-term hemodialysis patients after using dialysis fluid containing sodium acetate as the buffering agent (*149*). Hyperacetatemia has been associated with the development of dyslipoproteinemia and atherosclerosis, especially in some hemodialysis patients (*150*), and has also been implicated as a main cause of alcohol hangover headache recently (*151*). Moreover, increased serum acetate has been shown as a better marker of problem drinking among drunken drivers than ethanol (*152*). At present, the acetate level in the human patients is mainly monitored by measuring its level in blood. However, the results from this study suggested that urinary NAT can potentially become an effective biomarker of hyperacetatemia based on the correlation between serum acetate level and urinary NAT level, as well as the reaction mechanism of NAT biosynthesis. Because of the noninvasive nature of urine collection and the convenience of NAT detection (which doesn't require the derivatization procedure used in the measurement of acetate), measuring NAT might provide an alternative approach for evaluating the status of acetate metabolism and enable the early diagnosis of diseases and toxic effects associated with hyperacetatemia. This hypothesis will require further validation in various hyperacetatemic situations.

Taurine, with its numerous physiological functions, is a highly abundant free amino acid in the body. Its intracellular concentration ranges between 5-50 mM in many mammalian tissues (*141*). Because taurine is an end product of methionine metabolism pathway (*153*), its level can partially reflect the status of sulfur-containing amino acids *in vivo*. The protective effects of taurine against the ethanol-induced toxicities, such as hepatic steatosis and lipid peroxidation, have been revealed in several studies on taurine supplementation (*154*, *155*), and further proven by the studies of taurine depletion, in which increased susceptibility to ethanol-induced hepatic dysfunction was observed (*156*). The mechanism behind these observations has been largely attributed to the indirect interaction between taurine and ethanol through the membrane protection and antioxidant activities of taurine. Our results from this study provide the first evidence that taurine reacts directly with acetate, a major metabolite of ethanol, at physiologically relevant concentrations. Therefore, besides its indirect interaction with ethanol, taurine indeed can directly interfere with ethanol metabolism through the formation of NAT to remove excessive acetate. This conclusion is further supported by the observation of much higher activity of NAT biosynthesis in the kidney than other organs, which can facilitate the excretion of NAT into urine. Further studies will demonstrate whether the supplementation or the depletion of taurine will significantly affect the NAT production after ethanol exposure and whether taurine could be an effective antidote against the hyperacetatemia in the pathological conditions.

One of the aims of this study is to examine the role of CYP2E1 in the development of ethanol-induced steatosis since the previous studies yielded different conclusions on this

issue (*126-129*). Both histological and biochemical analysis of the responses of the wild-type and *Cyp2e1*-null mice to ethanol treatment in this study (Figure 2.2 and 2.3) indicated that, deficiency of CYP2E1 reduces the toxic effects of ethanol. The correlation of CYP2E1 genotype with higher NAT level in urine (Figure 2.4A-B) and higher acetate level in serum (Figure 2.7D) further supports the role of CYP2E1 in ethanol metabolism as an enzyme participating the oxidation of ethanol to acetaldehyde, especially in ethanol overdose. Interestingly, our conclusion on a contributing role of CYP2E1 in the ethanol-induced toxicity is consistent with the results from previous studies using comparable oral feeding method (*126, 127*), while different from other studies using intragastric infusion method, in which CYP2E1 deficiency failed to make a difference in terms of steatosis (*128, 129*). Even though the causes leading to these inconsistent observations on ethanol and CYP2E1 remain largely unknown, it has been suggested that the endotoxemia and the increased TNFα level associated with the intragastric infusion model might contribute to the differences among these studies (*127*).

The identification of NAT as a metabolite of ethanol and a biomarker of hyperacetatemia was mainly facilitated by the adoption of untargeted LC-MS-based metabolomic analysis in this study since the traditional bioanalysis approaches focusing on examining a single molecule or a defined cluster of molecules lack the capacity to detect unexpected or novel metabolites in complex biological matrices. Compared to previous metabolomics studies on ethanol intoxication (*131-133, 157*), our current study has further expanded the power of metabolomics in characterizing biochemical events using stable isotope labeling analysis. Because of the mass difference between unlabeled compound and its stable

isotope-labeled counterpart, the metabolic fates of exogenous compounds can be effectively tracked through the MS-based metabolomic analysis of biological samples in the loadings plot of MDA model, as shown by the identification of ethanol metabolites in this study (Figure 2.6A) and the identification of novel acetaminophen (APAP) metabolites in our recent study on the toxicity-related APAP metabolites (*158*). In this study, the stable isotope labeling approach also conveniently distinguished the endogenous NAT from the exogenous NAT after deuterated ethanol and acetate treatment (Figure 2.6C and 2.7F). Because of its effectiveness in the identification of metabolite and characterization of metabolic pathway, stable isotope labeling will have broad application in the MS-based metabolomics.

Overall, the combination of LC-MS-based metabolomics, stable isotope labeling, animal modeling, and *in vivo* and *in vitro* biochemical analysis in this study enabled the unambiguous identification of NAT as a novel metabolite of ethanol formed by the unreported enzymatic reaction between acetate and taurine. Ethanol-induced hyperacetatemia, which is partially contributed by CYP2E1-mediated ethanol metabolism, is likely the main cause of increased NAT level in urine. The value of NAT as the biomarker of diseases, such as ethanol-induced tissue injury or hemodialysis-related toxicity, requires further investigation.

Table 1 Urinary metabolite markers of ethanol treatment

Markers	RT (min)	$[M-H]^-$	Formula	Identity
I	0.35	166.0174	$C_4H_8NO_4S^-$	NAT
II	1.97	230.9963	$C_8H_7O_6S^-$	4-hydroxyphenylacetic acid
III	0.68	221.0661	$C_8H_{13}O_7^-$	ethyl glucuronide
IV	0.33	124.9909	$C_2H_5O_4S^-$	ethyl sulfate
V	5.39	317.1592	$C_{15}H_{25}O_7^-$	N.D.
VI	5.30	329.1600	$C_{16}H_{25}O_7^-$	N.D.
VII	5.62	319.1756	$C_{15}H_{27}O_7^-$	N.D.
VIII	5.24	317.1598	$C_{15}H_{25}O_7^-$	N.D.
IX	1.97	151.0395	$C_8H_7O_3^-$	in-source fragment of **II**
X	2.32	308.0982	$C_{11}H_{18}NO_9^-$	*N*-acetylneuraminic acid

Table 1 The process of identifying urinary metabolites responsive to ethanol treatment was described in the *Materials and Methods* and *Results*. The most prominent markers revealed by the loadings plot of PLS-DA model (labeled in Figure 2.3B) were further investigated by accurate mass measurement, MS/MS fragmentation. Retention time (RT) of each marker was its elution time from a 10-min run in a C_{18} UPLC column. The identities of markers **I**, **II**, **III, IX,** and **X** were confirmed by the comparisons with authentic standards (N.D., not determined).

Figure 2.1

Figure 2.1 Comparison of the feeding tubes for semi-solid ethanol diet and liquid ethanol diet. The customized feeding tube for semi-solid ethanol diet (**a**) and the standard feeding tube for liquid ethanol diet (**b**) are shown above.

Figure 2.2A

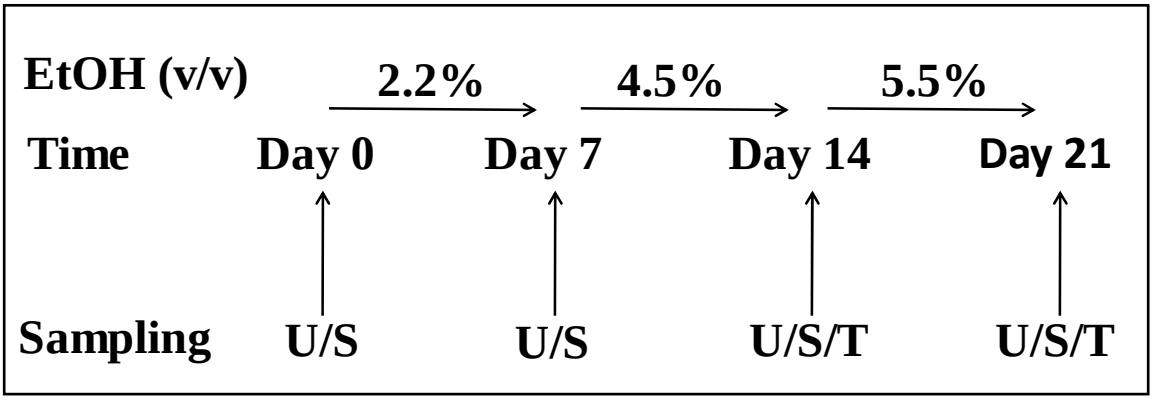

Figure 2.2A Schedule of ethanol treatment and sample collection. Both WT and KO mice were fed with either control (Ctl) or ethanol (EtOH) diet for 21 days. The concentration of ethanol was increased weekly from 2.2% (v/v) to 4.5% (v/v) and finally 5.4% (v/v). Urine (U) and serum (S) samples were collected once a week. Tissues (T) were harvested at day 14 and 21.

Figure 2.2B

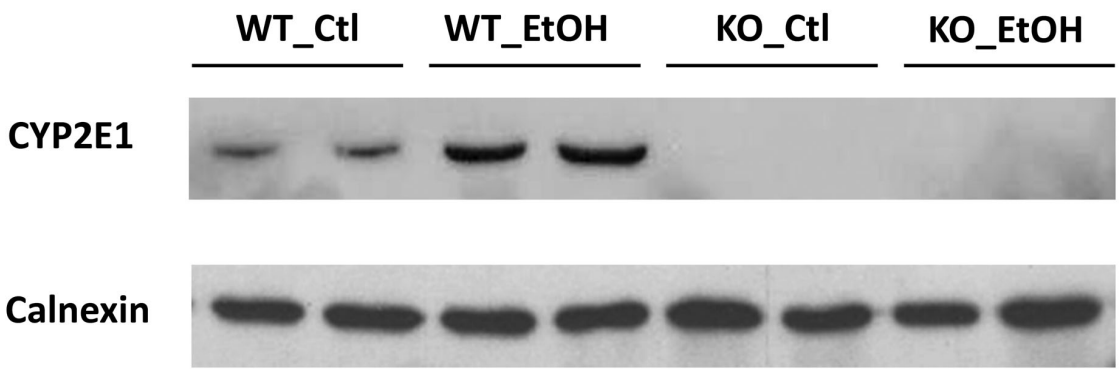

Figure 2.2B Influence of ethanol treatment on CYP2E1 protein level of wild-type (WT) and *Cyp2e1*-null (KO) mice. Western blot of CYP2E1 protein expression after 21-day feeding of control or ethanol diet showed CYP2E1 was induced several fold in WT mice after ethanol consumption, but was undetectable in KO mice.

Figure 2.2C

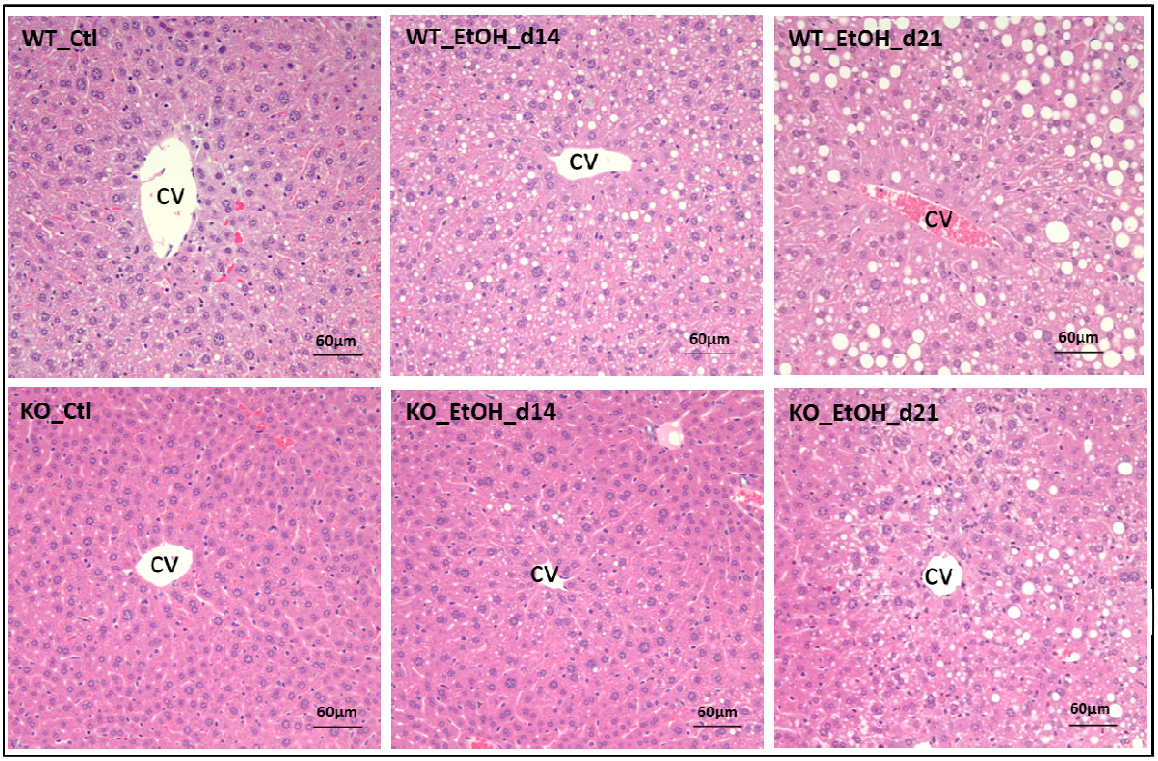

Figure 2.2C Influence of ethanol treatment on liver histology of wild-type (WT) and *Cyp2e1*-null (KO) mice fed with control or ethanol diet. The location of hepatic central vein (CV) was marked.

Figure 2.3A

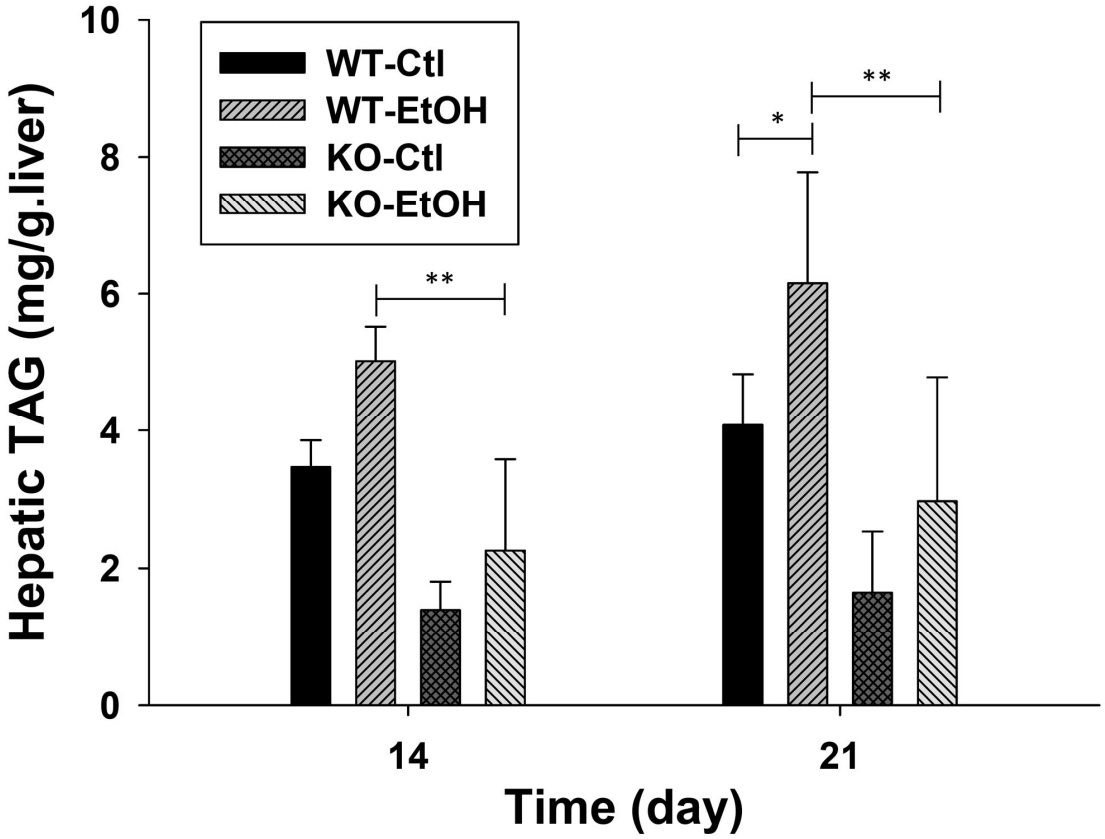

Figure 2.3A Triacylglycerol (TAG) level in the liver of wild-type (WT) and *Cyp2e1*-null (KO) mice after fed with control or ethanol diets for 21 days. Values were presented as mean ± S.D (n=8). * ($p<0.05$), and ** ($p<0.01$).

Figure 2.3B

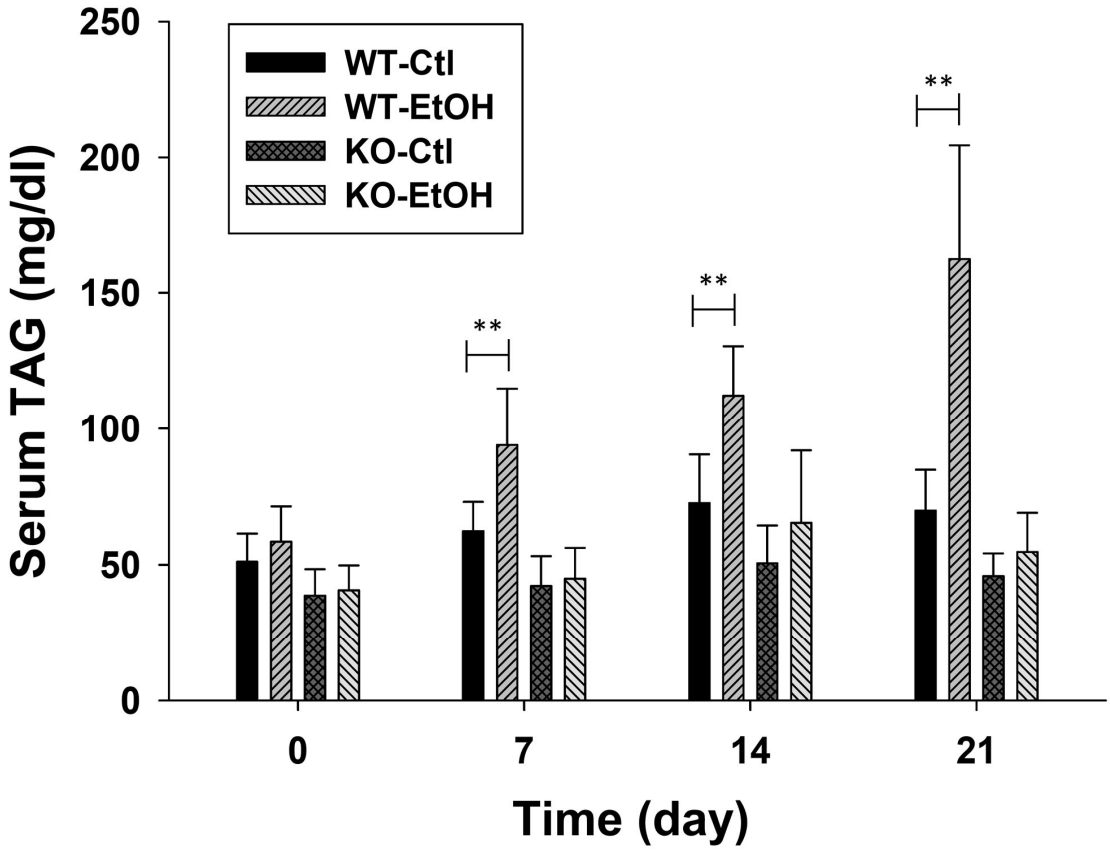

Figure 2.3B TAG level in the serum of wild-type (WT) and *Cyp2e1*-null (KO) mice after fed with control or ethanol diet for 21 days. Values were presented as mean ± S.D (n=8). * ($p<0.05$), and ** ($p<0.01$).

Figure 2.3C

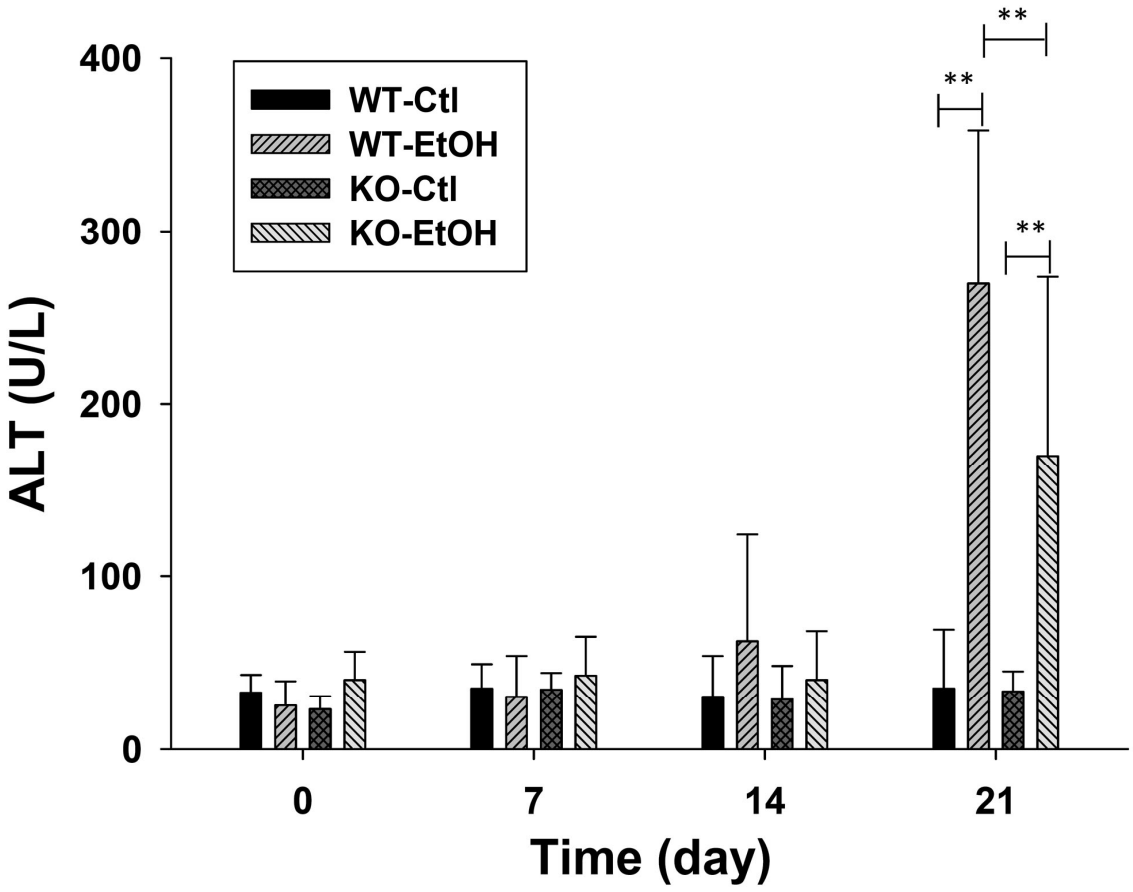

Figure 2.3C Serum alanine transaminase (ALT) activity of wild-type (WT) and *Cyp2e1*-null (KO) mice after fed with control or ethanol diet for 21 days. Values were presented as mean ± S.D (n=8). * ($p<0.05$), and ** ($p<0.01$).

Figure 2.3D

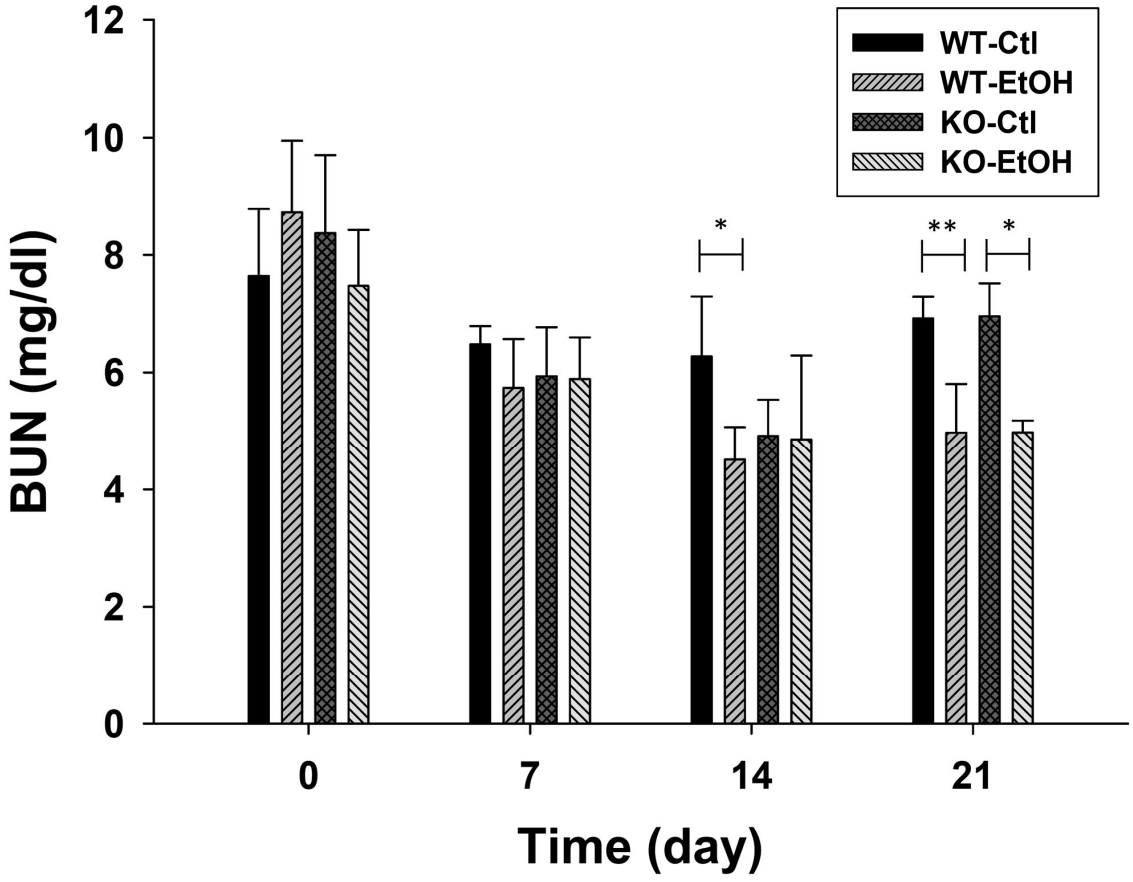

Figure 2.3D Blood urea nitrogen (BUN) level of wild-type (WT) and *Cyp2e1*-null (KO) mice after fed with control or ethanol diet for 21 days. Values were presented as mean ± S.D (n=8). * ($p<0.05$), and ** ($p<0.01$).

Figure 2.4A

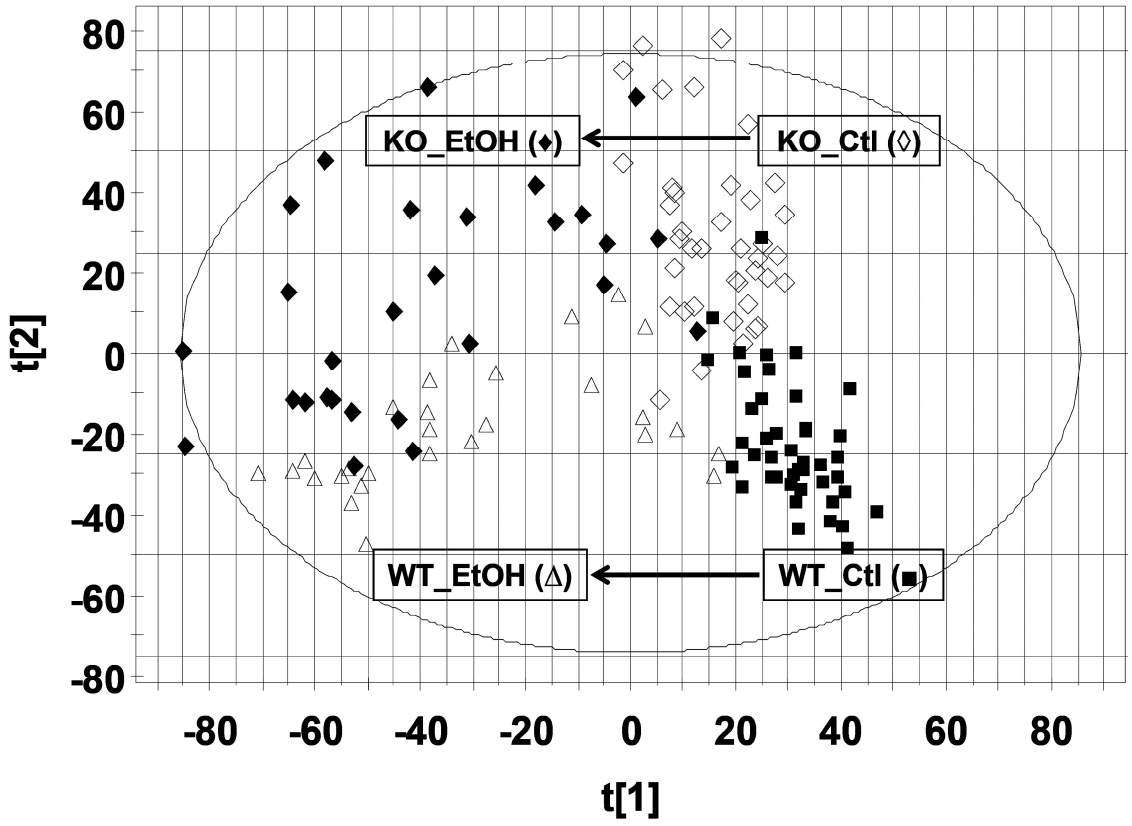

Figure 2.4A The scores plot of a PLS-DA model on urine samples from the wild-type and *Cyp2e1*-null mice fed with control and ethanol diets. All samples from the wild-type and *Cyp2e1*-null mice with no ethanol exposure were labeled as WT_Ctl (■) and KO_Ctl (◊), respectively, while the samples from both mouse lines with ethanol treatment were labeled as WT_EtOH (Δ) and KO_EtOH (♦), respectively, which include the samples from 7-day, 14-day and 21-day ethanol treatment. The *t*[1] and *t*[2] values represent the scores of each sample in the principal component 1 and 2, respectively.

Figure 2.4B

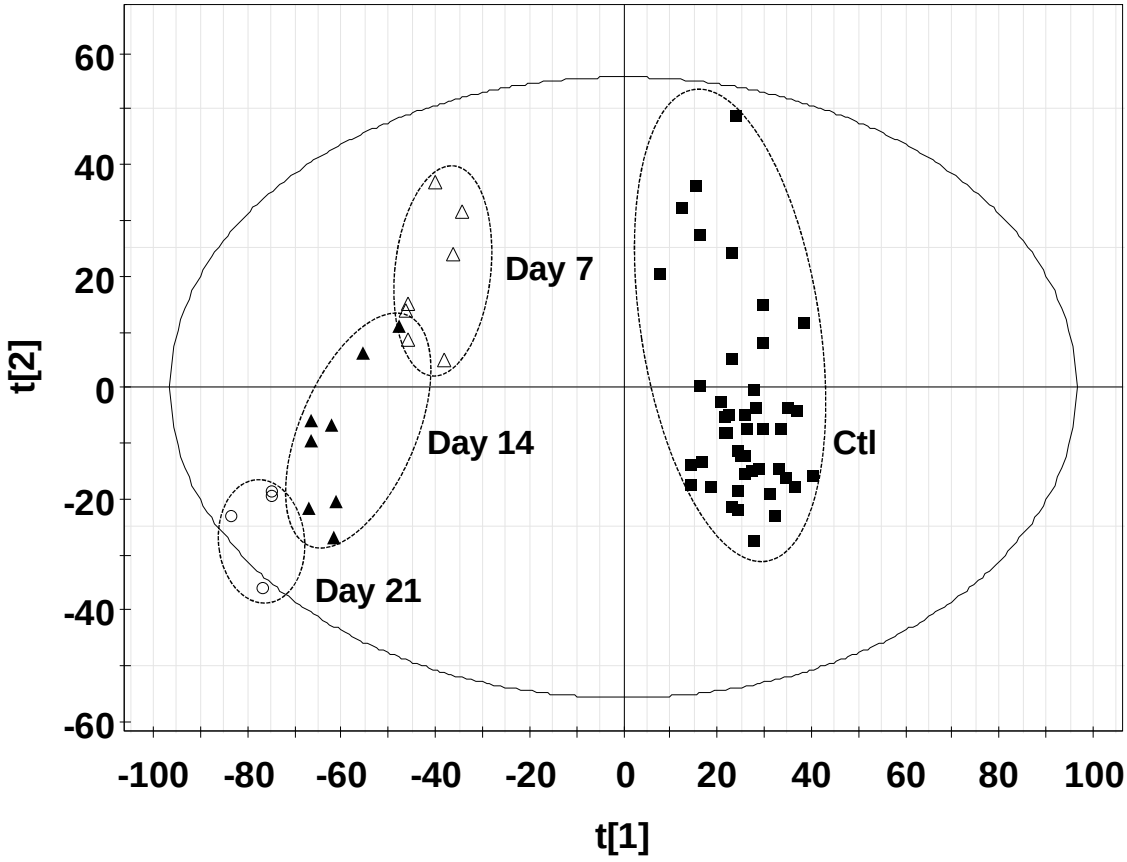

Figure 2.4B The scores plot of a PLS-DA model on urine samples from the wild-type mice fed with control and ethanol diets. Conditions of LC-MS measurement and procedures of data processing and analysis were described in the *Materials and Methods*. All samples from the mice with no ethanol exposure were labeled as Ctl (■) while the samples from ethanol treatment were labeled according to the time points of sample collection, which are day 7 (Δ), 14 (▲), and 21 (○). The *t*[1] and *t*[2] values represent the scores of each sample in the principal component 1 and 2, respectively. Fitness (R^2) and prediction power (Q^2) of this PLS-DA model are 0.64 and 0.43, respectively. The model was validated through the recalculation of R^2 and Q^2 values after the permutation of sample identities.

Figure 2.4C

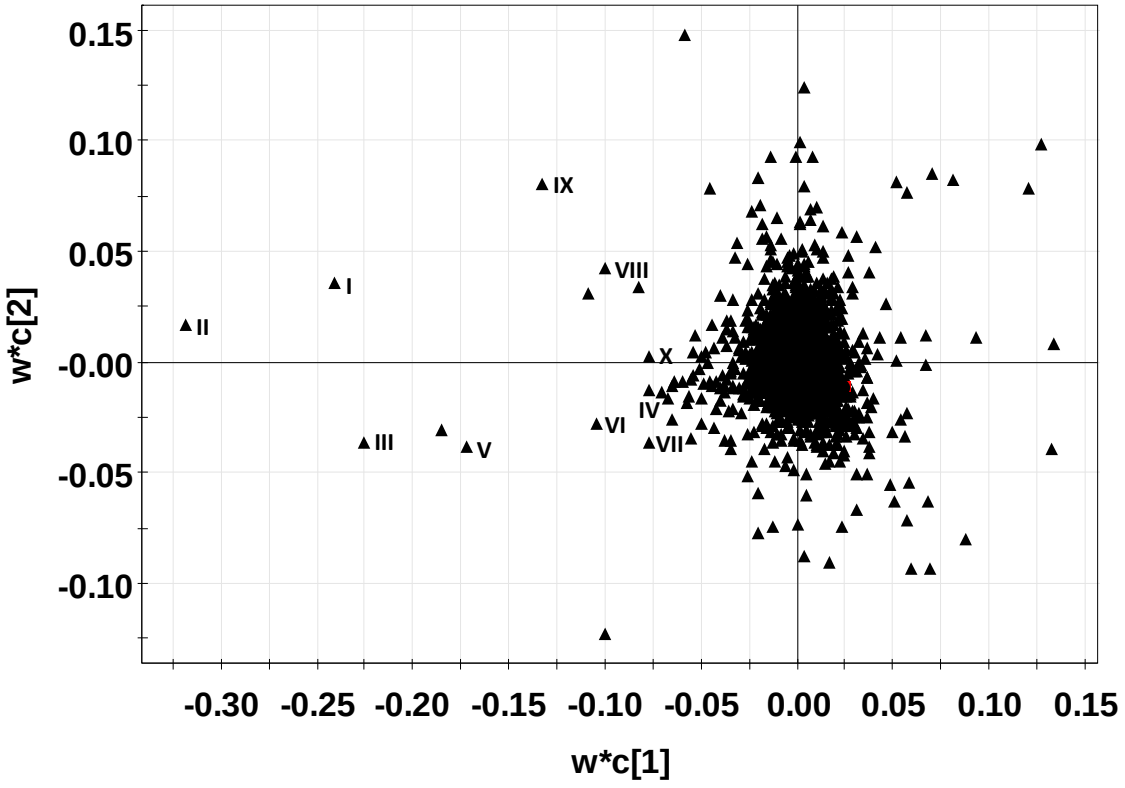

Figure 2.4C The loadings plot of urinary ions contributing to the classification of urine samples from the wild-type mice treated with control and ethanol diet. The *w*c*[1] and *w*c*[2] values represent the contributing weights of each ion to the principal components 1 and 2 of the PLS-DA model, respectively. Major urinary ions (**I–X**) induced by ethanol treatment in the wild-type mice were labeled.

Figure 2.4D

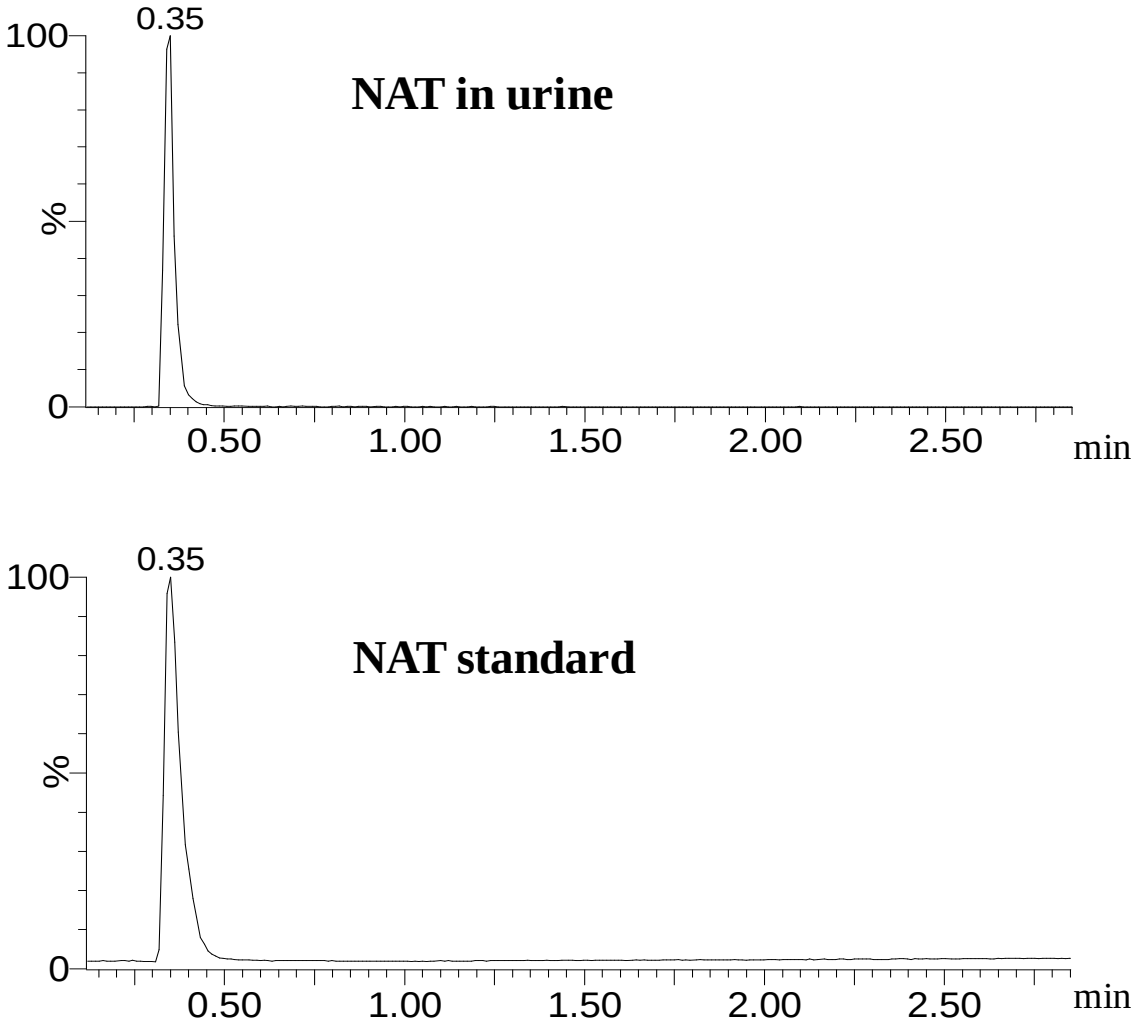

Figure 2.4D The extracted chromatograms of NAT in urine (**I**) and NAT standard.

Figure 2.4E

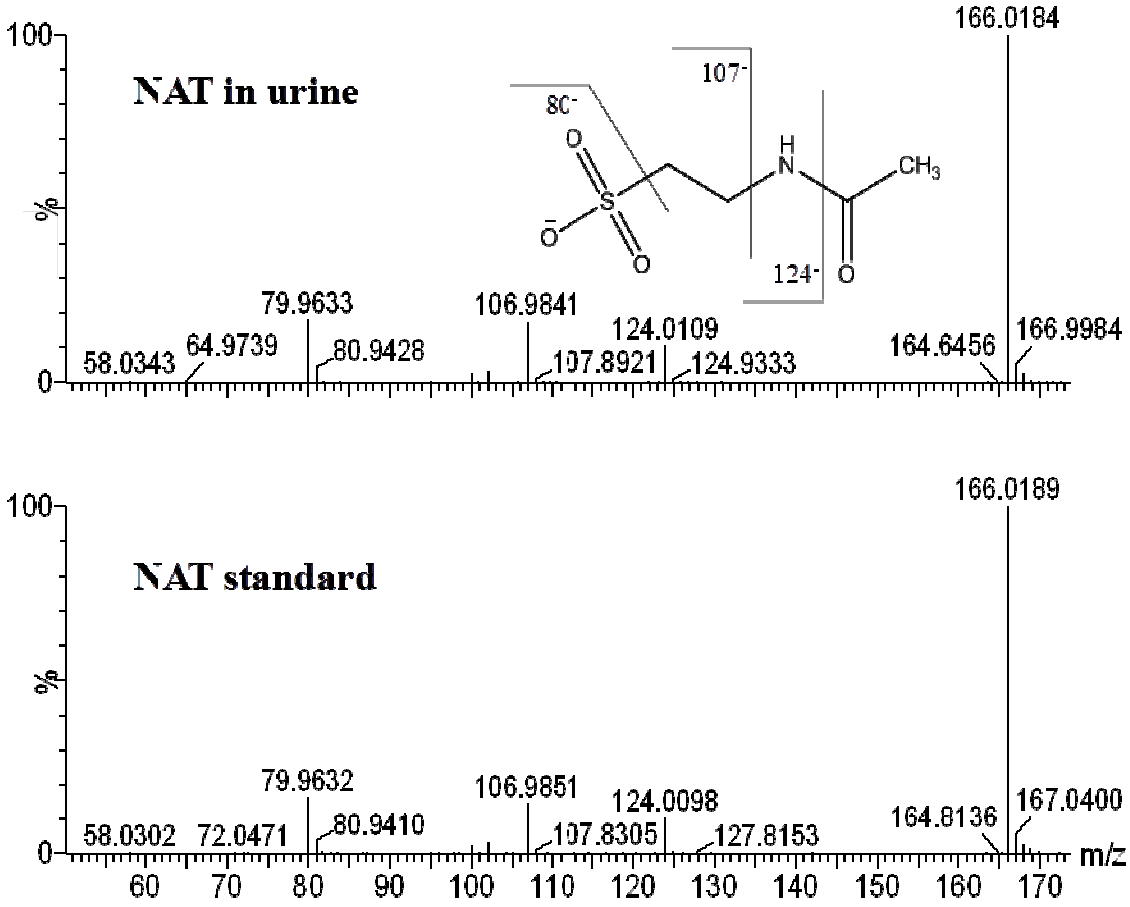

Figure 2.4E Representative MS/MS fragmentation spectra of NAT in urine (**I**) and NAT standard.

Figure 2.5A

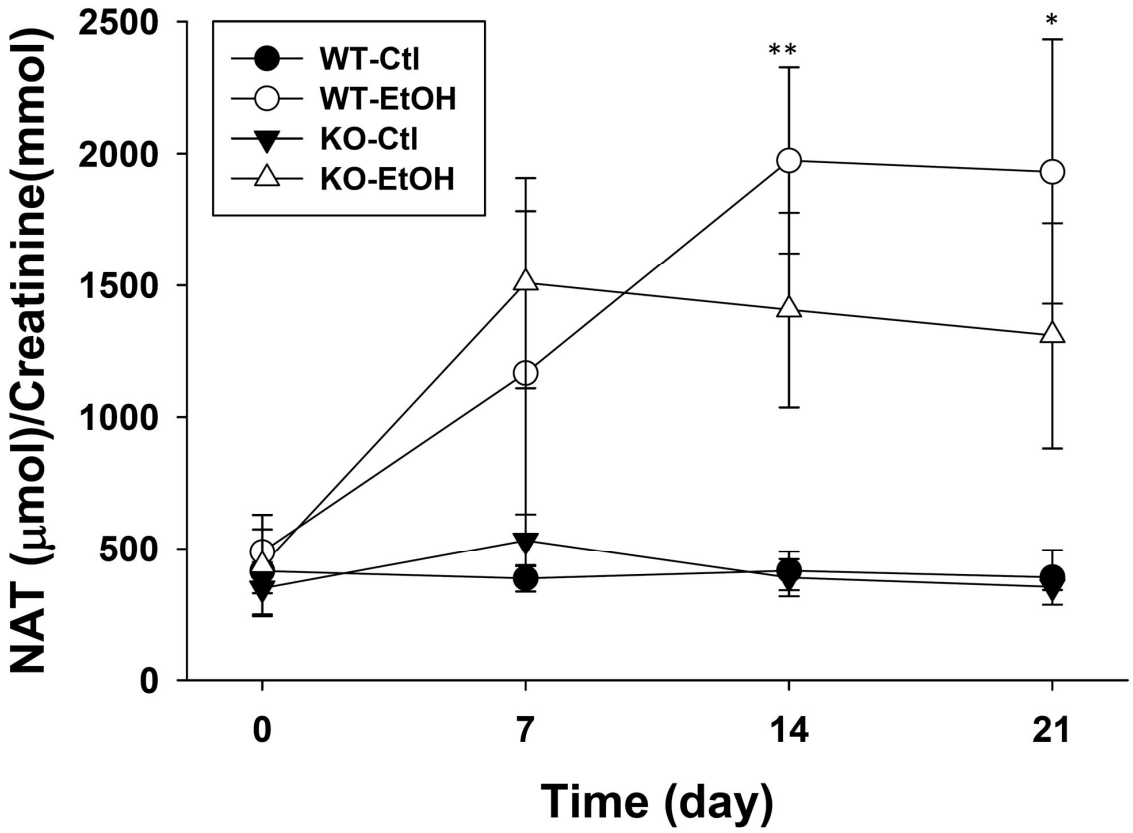

Figure 2.5A Quantitation of urinary biomarkers of ethanol exposure---NAT. Urinary concentrations of NAT in both wild-type (WT) and *Cyp2e1*-null (KO) mice during the 21-day ethanol treatment were calculated as the molar ratio to creatinine. Values were presented as mean ± S.D (n=8). * (p<0.05) and ** (p<0.01) indicate statistical significance between WT and KO samples at the same time point.

Figure 2.5B

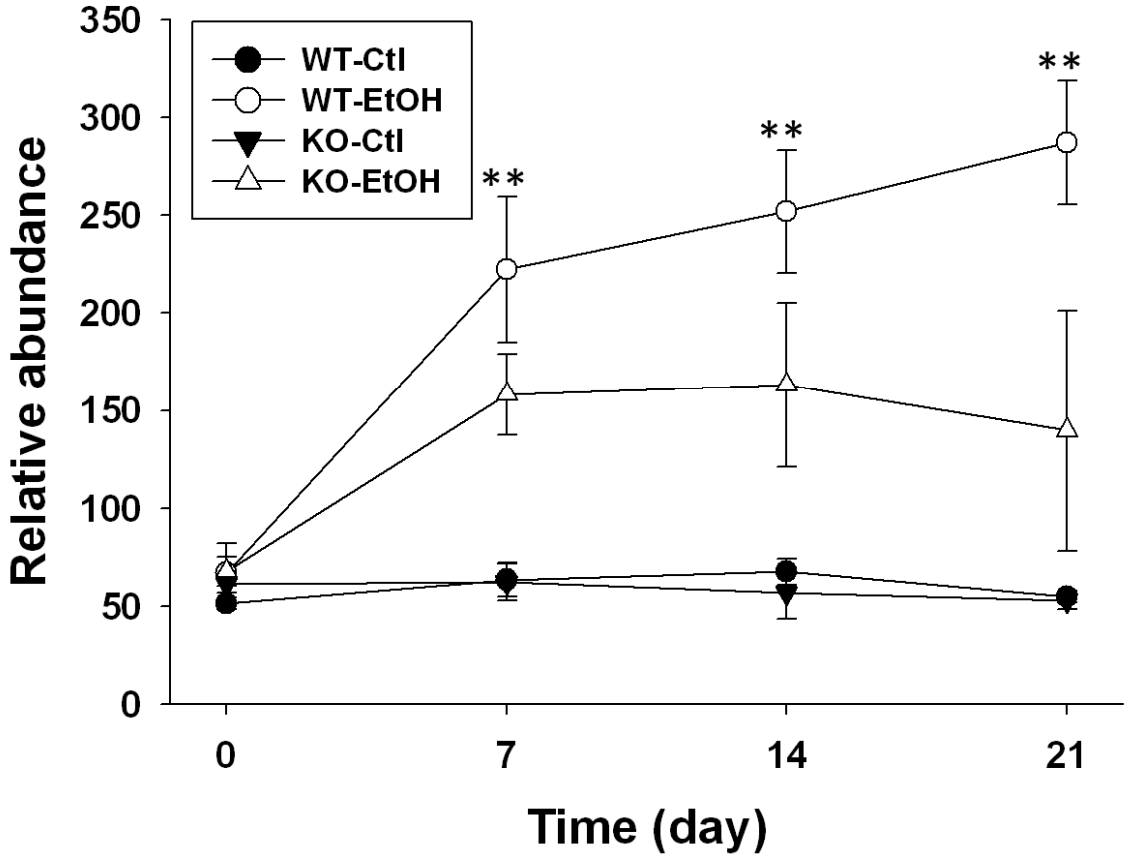

Figure 2.5B Relative abundance of urinary biomarkers of ethanol exposure---NAT in the wild-type (WT) and *Cyp2e1*-null (KO) mice during the 21-day ethanol treatment. The abundance of urinary NAT was calculated as the 10,000 fold of the ratio between the single ion count (SIC) of NAT and the total ion count (TIC) of a urine sample detected by mass spectrometer (Relative abundance = 10000 × SIC/TIC). Values were presented as mean ± S.D (n=8). * (p<0.05) and ** (p<0.01) indicate statistical significance between WT and KO samples at the same time point.

Figure 2.5C

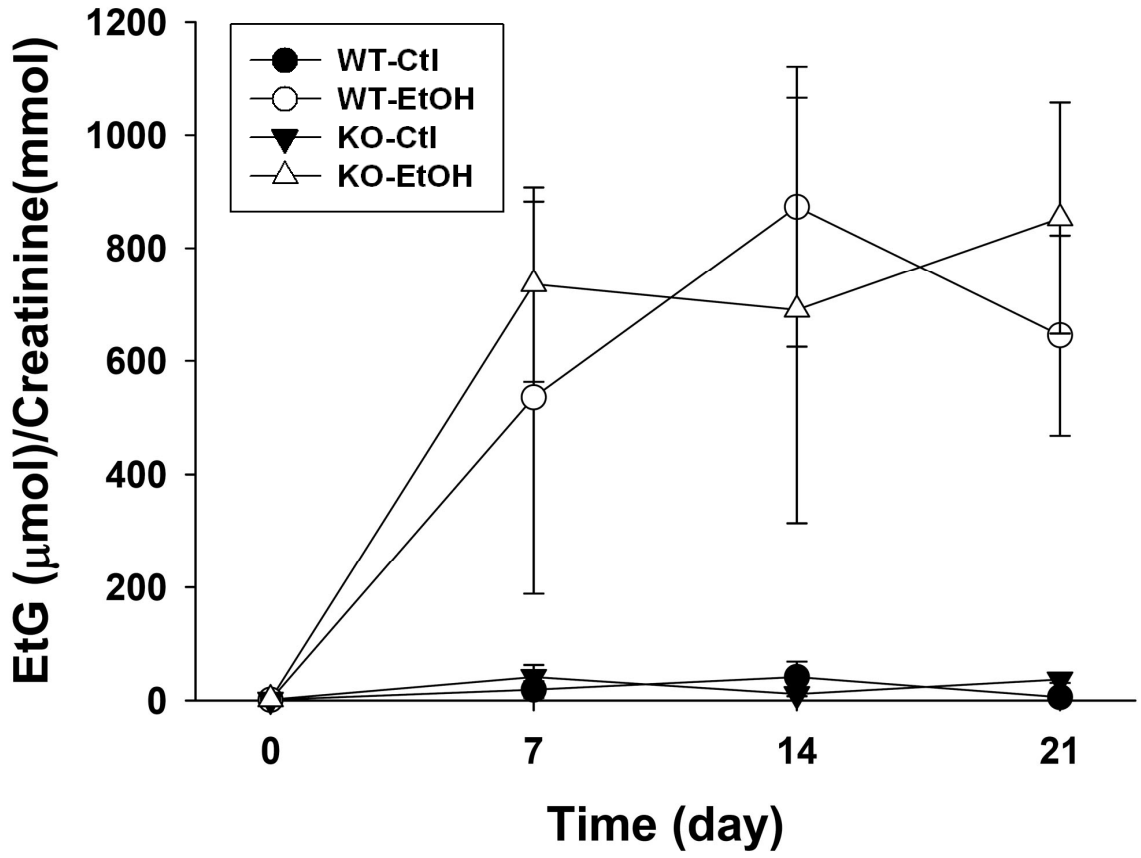

Figure 2.5C Quantitation of urinary biomarkers of ethanol exposure---EtG. Urinary concentrations of EtG in both wild-type (WT) and *Cyp2e1*-null (KO) mice during the 21-day ethanol treatment were calculated as the molar ratio to creatinine.

Figure 2.5D

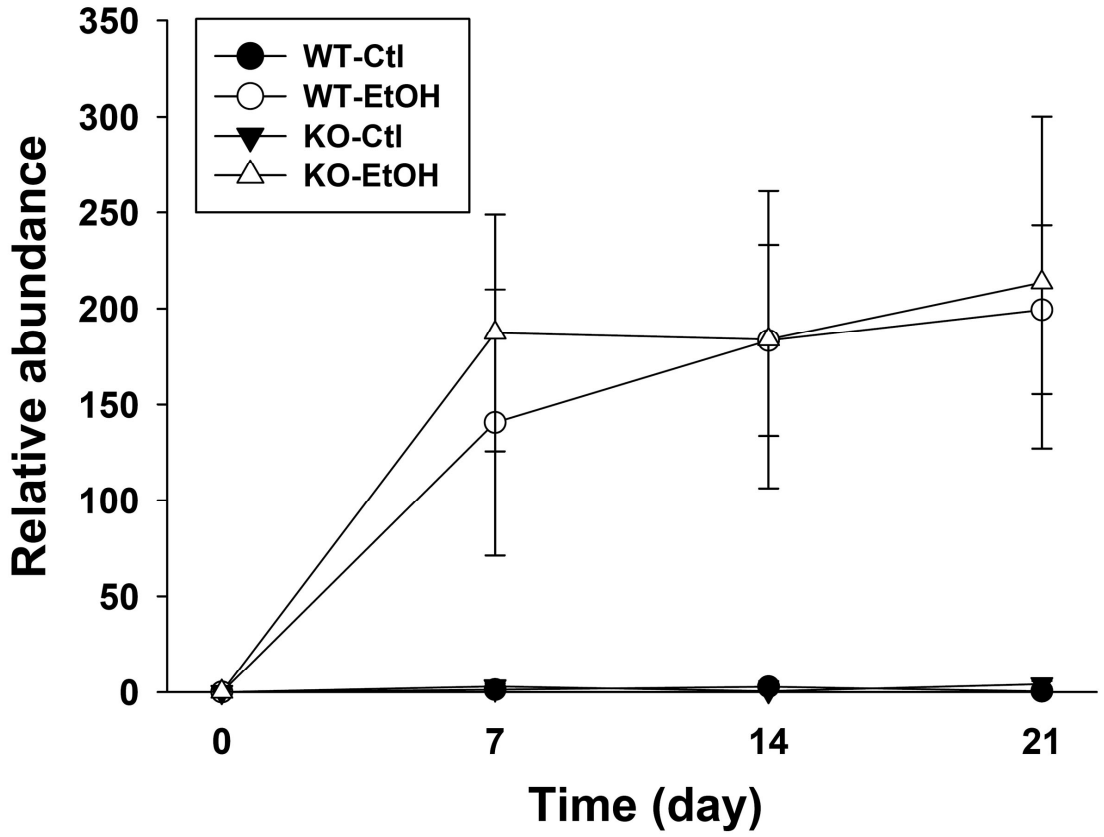

Figure 2.5D Relative abundance of urinary biomarkers of ethanol exposure---EtG in the wild-type (WT) and *Cyp2e1*-null (KO) mice during the 21-day ethanol treatment. The abundance of urinary EtG was calculated as the 10,000 fold of the ratio between the single ion count (SIC) of EtG and the total ion count (TIC) of a urine sample detected by mass spectrometer (Relative abundance = 10000 × SIC/TIC). Values were presented as mean ± S.D (n=8). * (p<0.05) and ** (p<0.01) indicate statistical significance between WT and KO samples at the same time point.

Figure 2.6A

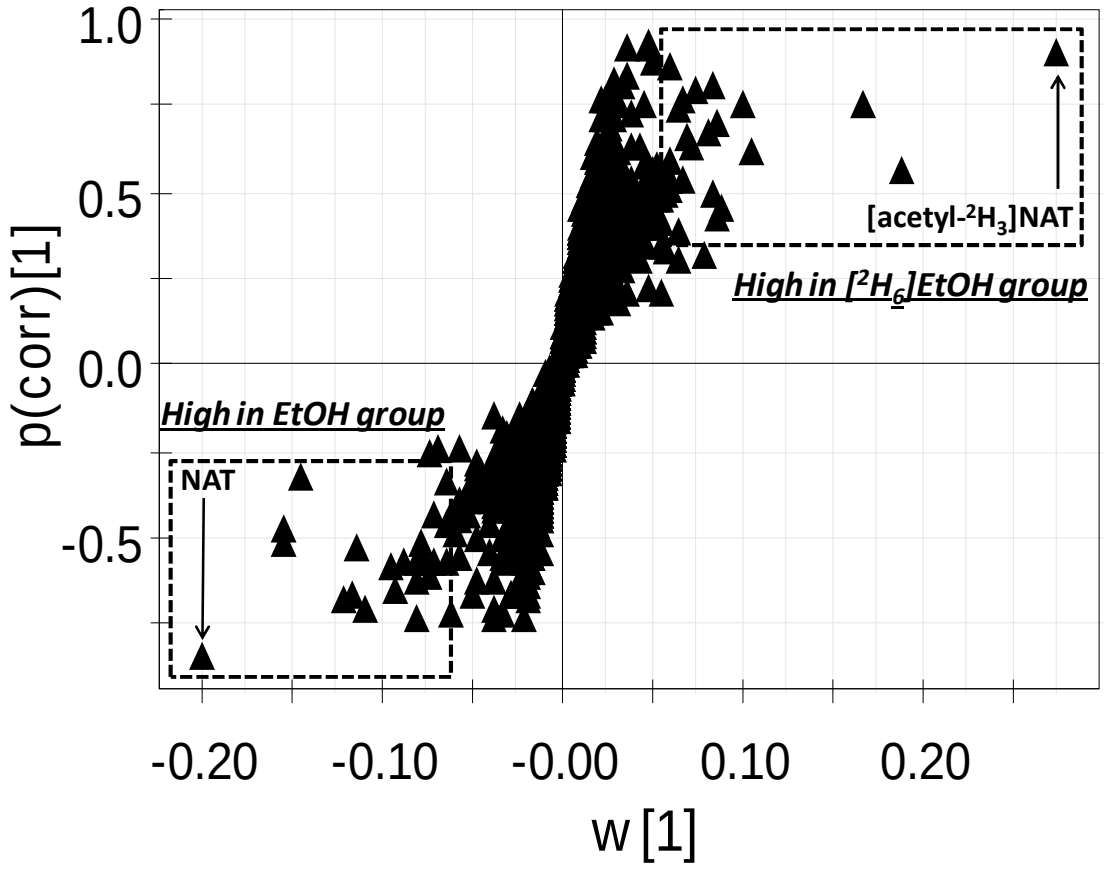

Figure 2.6A The S-plot of urinary ions from an OPLS analysis of LC-MS data from the wild-type mice treated with unlabeled and deuterated ethanol. The diet containing 2.2% unlabeled ethanol or deuterated ethanol ($[^2H_6]$-ethanol) was fed to the wild-type mice for 7 days as described in the *Materials and Methods*. NAT and its isotopic counterpart, [acetyl-$^2H_3$]NAT, are marked.

Figure 2.6B

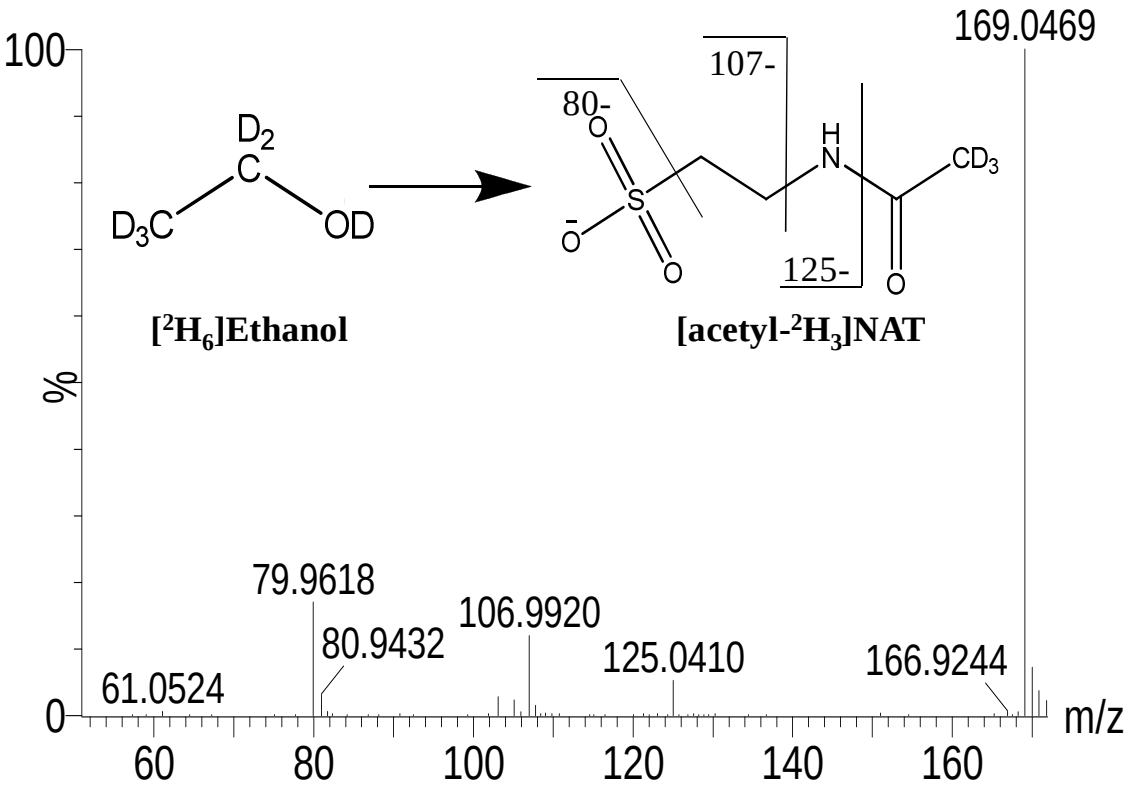

Figure 2.6B Representative MS/MS spectrum of [acetyl-$^{2}H_3$]NAT in the urine of wild-type mice treated with deuterated ethanol. The fragmentation pattern of [acetyl-$^{2}H_3$]NAT from deuterated ethanol was interpreted in the inlaid diagram.

Figure 2.6C

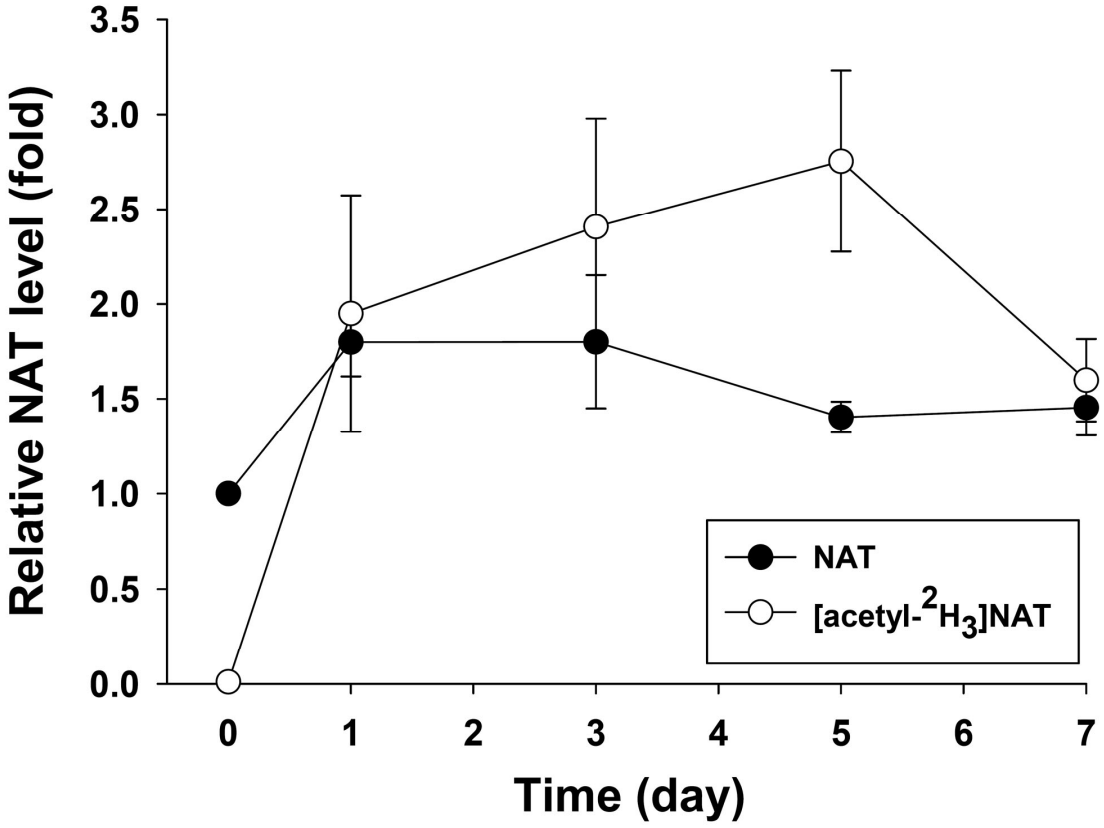

Figure 2.6C Relative abundance of NAT and [acetyl-$^2H_3$]NAT during the 7-day exposure of deuterated ethanol. The ion intensity of unlabeled NAT in the urine samples of wild-type mice prior to the exposure of deuterated ethanol was arbitrarily set as 1.

Figure 2.6D

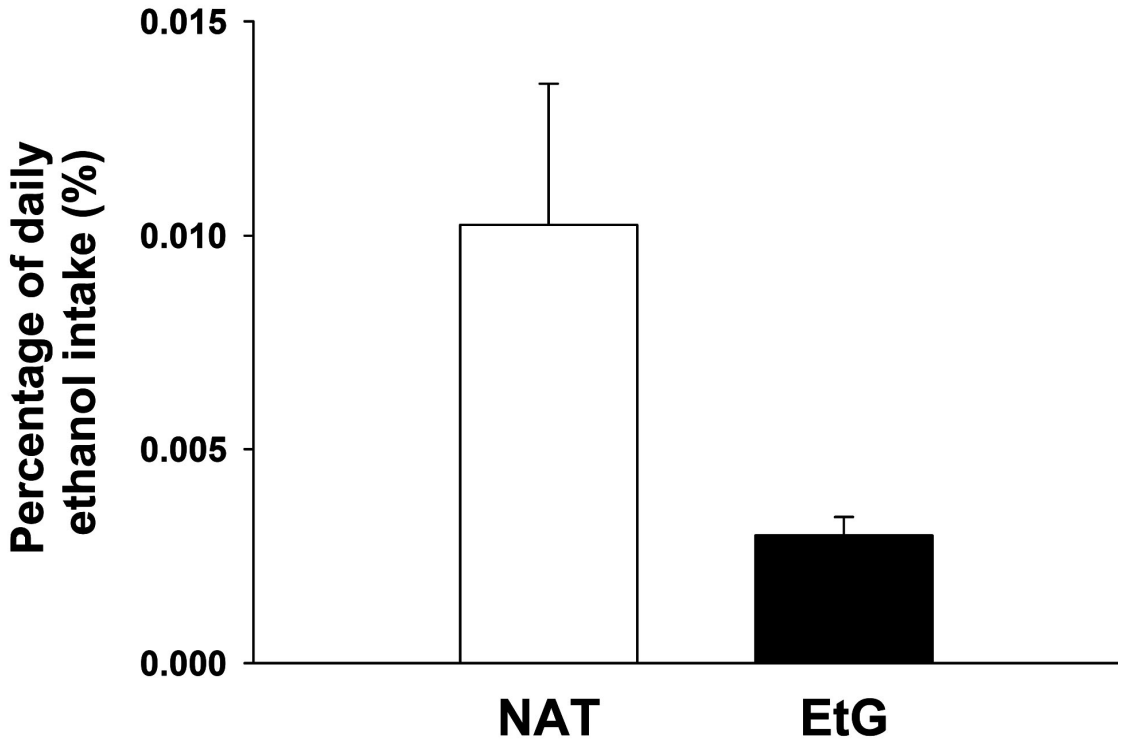

Figure 2.6D NAT as a minor metabolite of ethanol. The amounts of daily excretion of NAT and EtG in urine were calculated as the percentage of daily ethanol intake through an equal-molar conversion.

Figure 2.7A

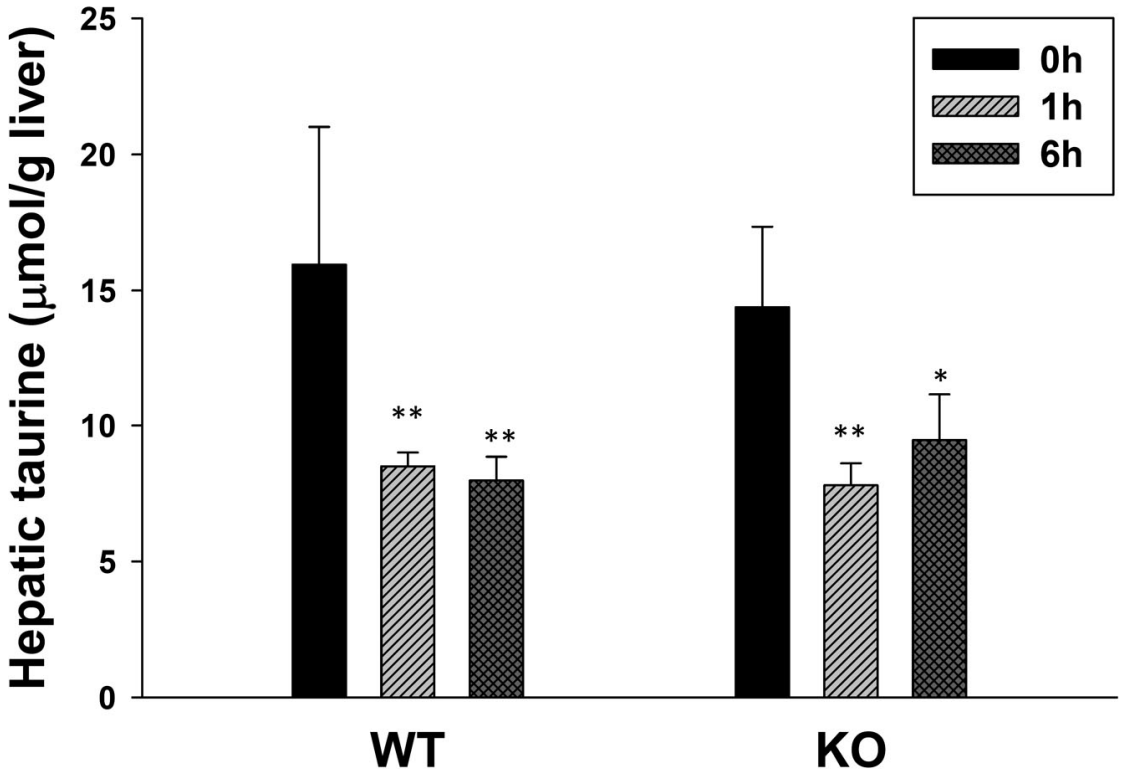

Figure 2.7A Hepatic taurine level. Levels of taurine as one of the potential precursors of NAT in the wild-type (WT) and the *Cyp2e1*-null (KO) mice after i.p. injection of 4 g/kg ethanol were measured by the LC-MS methods described in the *Materials and Methods*. Values were presented as mean ± S.D (n=4). * (p<0.05) and ** (p<0.01) indicate statistical significance between post- and before-ethanol treatments.

Figure 2.7B

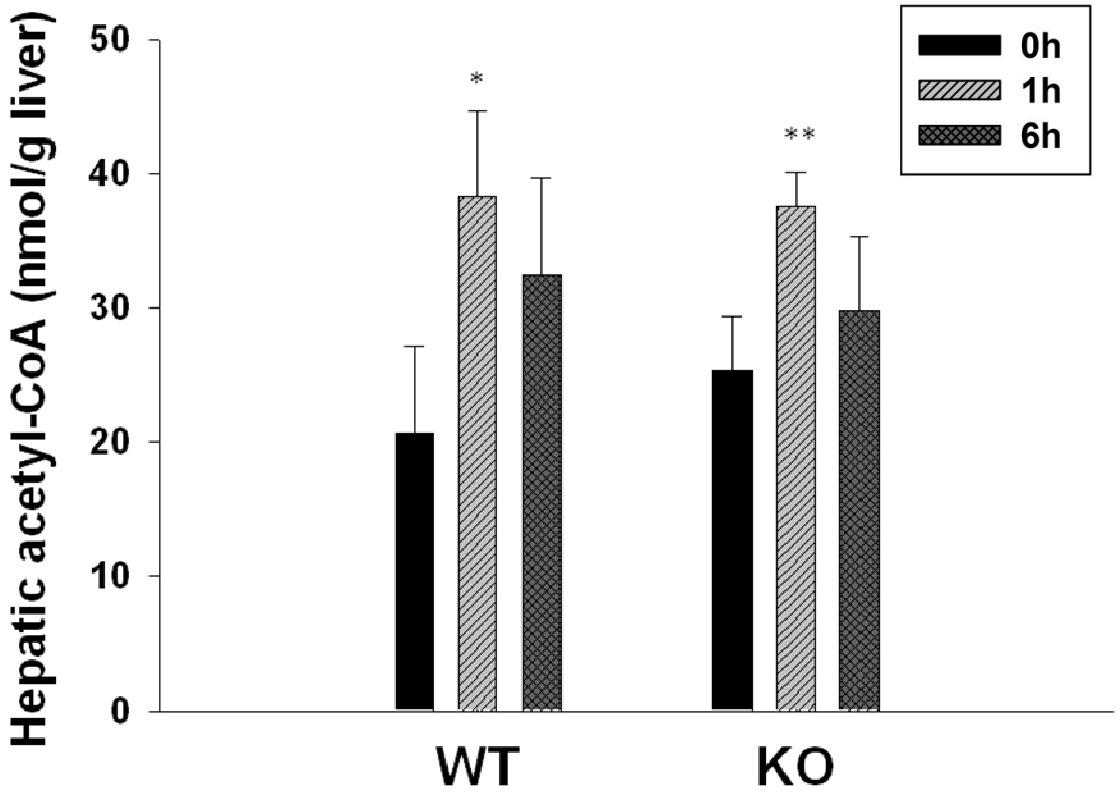

Figure 2.7B Hepatic acetyl-CoA level. Values were presented as mean ± S.D (n=4). * (p<0.05) and ** (p<0.01) indicate statistical significance between post- and before-ethanol treatments.

Figure 2.7C

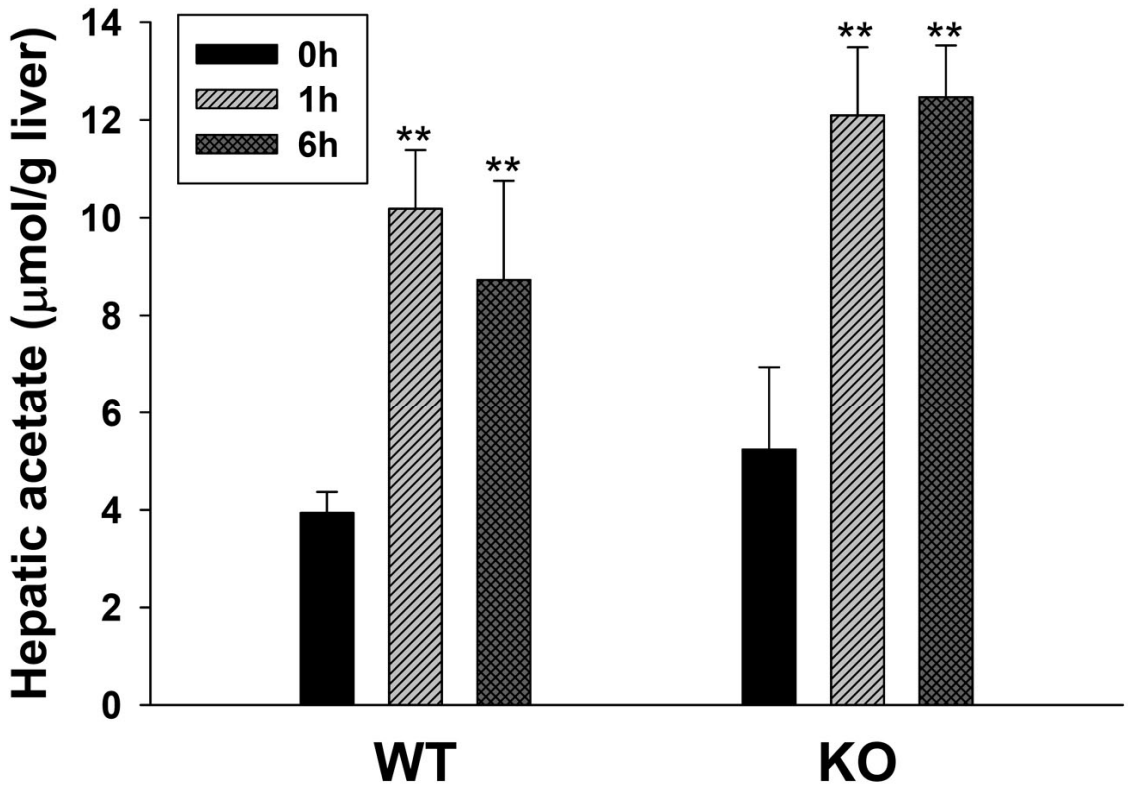

Figure 2.7C Hepatic acetate level. Values were presented as mean ± S.D (n=4). * (p<0.05) and ** (p<0.01) indicate statistical significance between post- and before-ethanol treatments.

Figure 2.7D

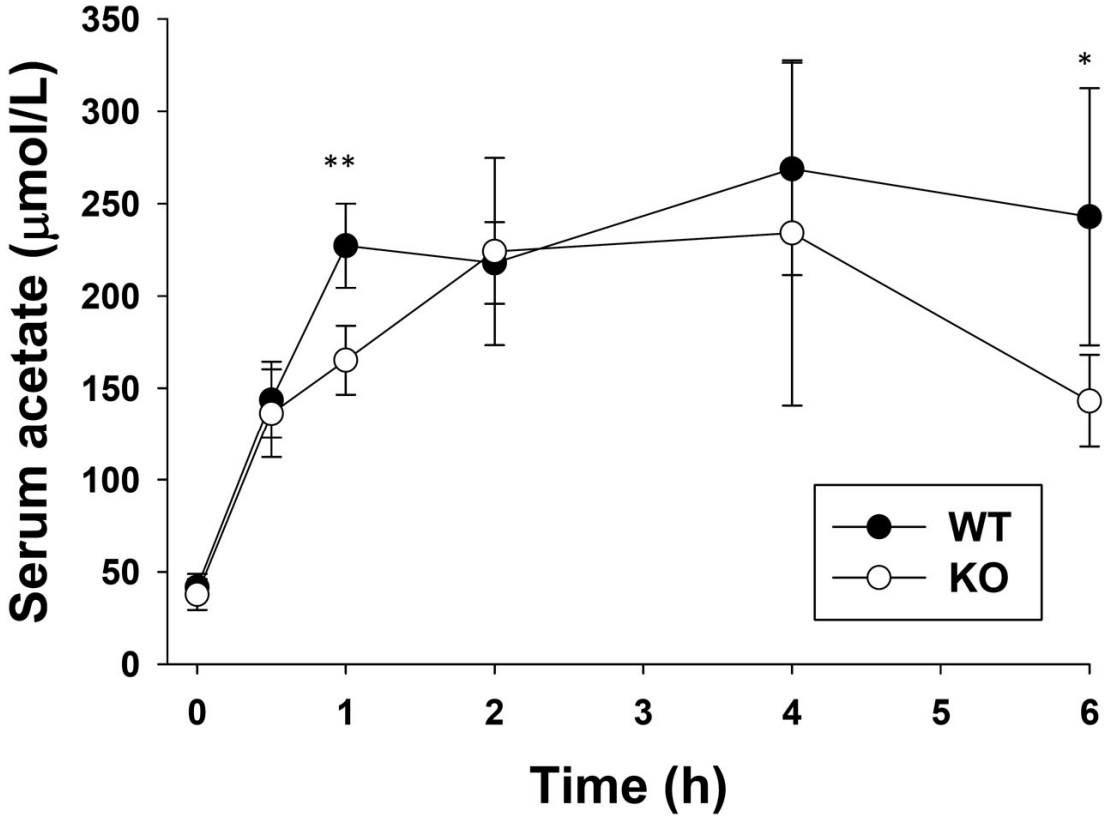

Figure 2.7D Serum acetate level. Values were presented as mean ± S.D (n=4). * (p<0.05) and ** (p<0.01) indicate statistical significance between WT and KO samples at the same time point.

Figure 2.7E

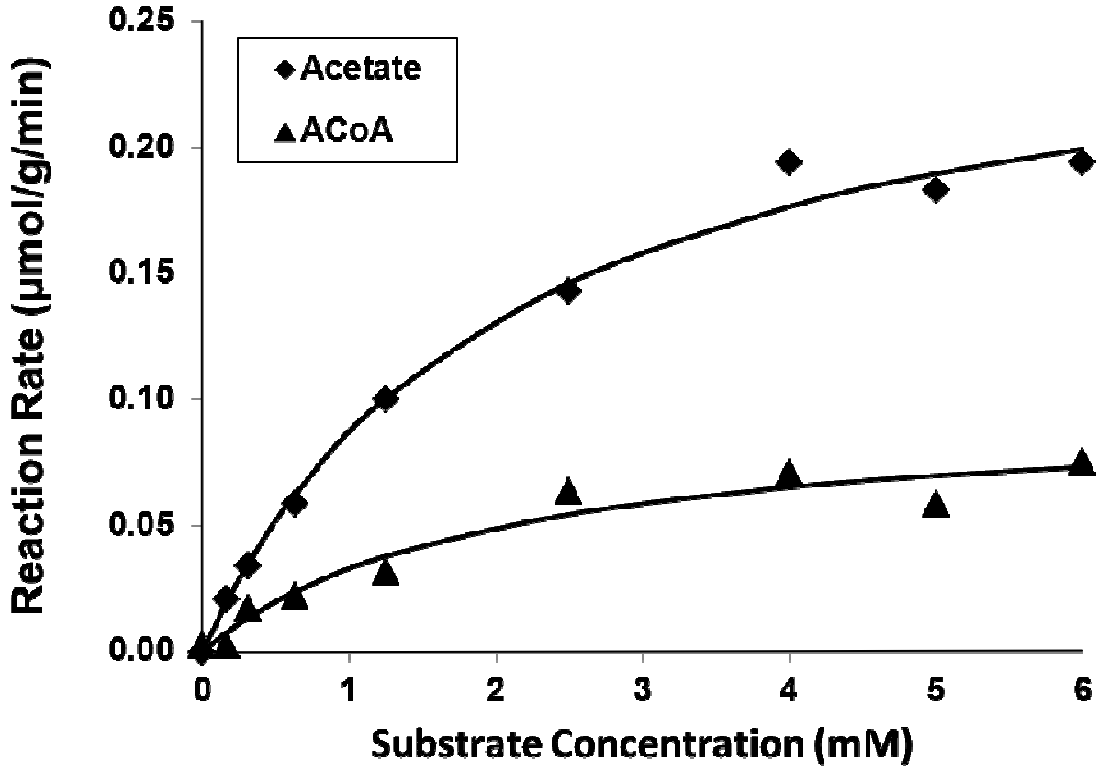

Figure 2.7E Enzyme kinetics of NAT biosynthesis *in vitro*. NAT biosynthesis was conducted by incubating liver homogenate with 20 mM taurine and various concentrations of acetate or acetyl-CoA.

Figure 2.7F

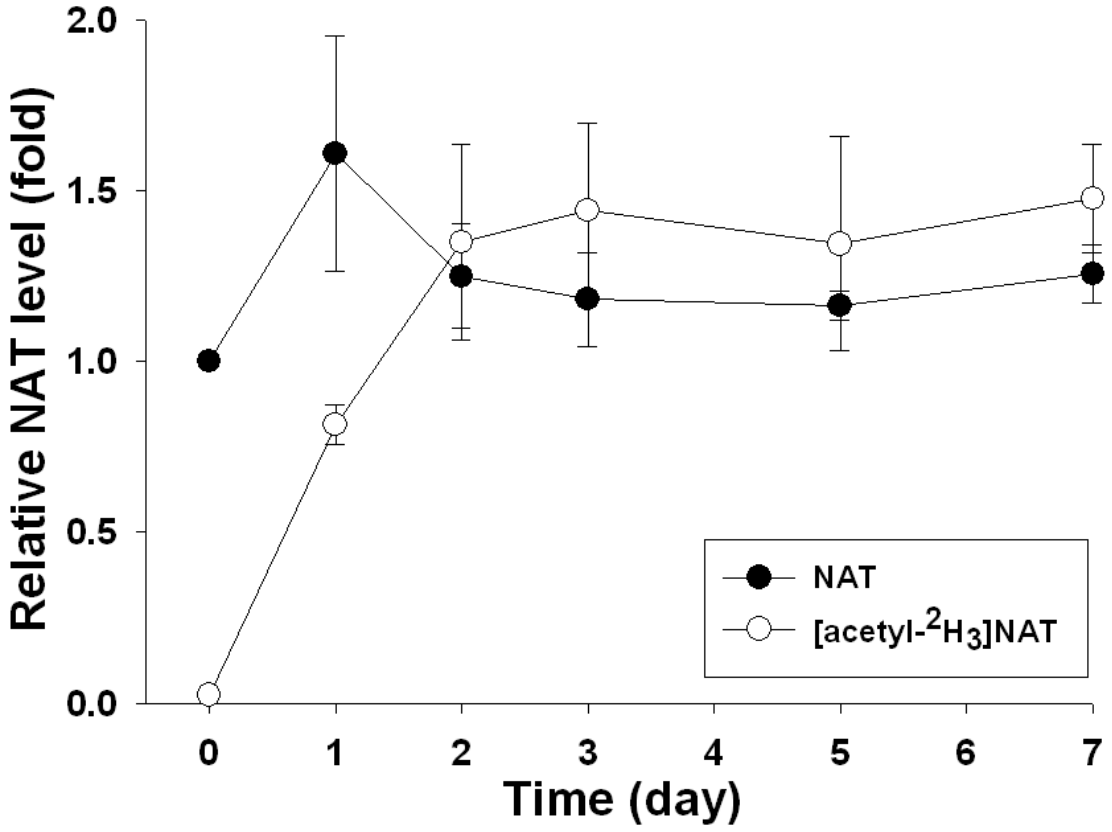

Figure 2.7F Relative abundance of NAT and [acetyl-$^2H_3$]NAT during the 7-day exposure of deuterated acetate. The WT mice were fed with the diet containing 1.1% deuterated acetate ([$^2H_4$]-acetate) for 7 days. The ion intensity of unlabeled NAT in the urine samples of WT mice prior to the exposure of deuterated acetate was arbitrarily set as 1.

Figure 2.8A

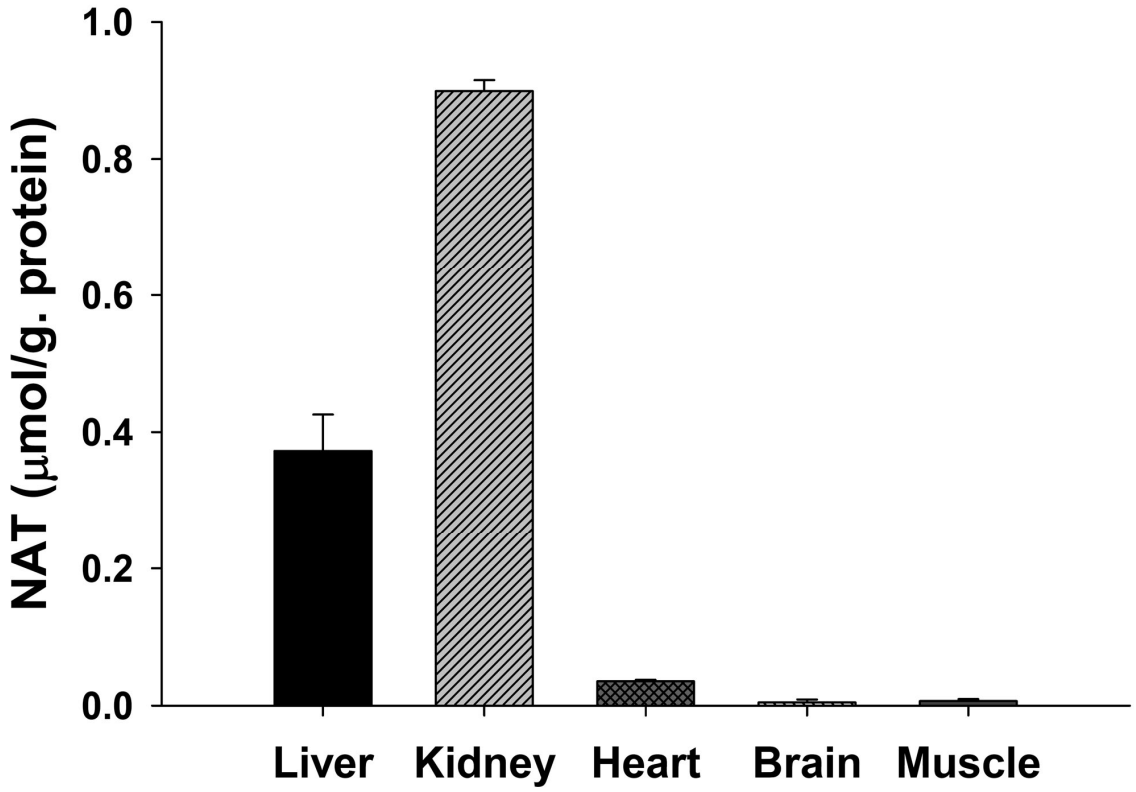

Figure 2.8A NAT biosynthesis by liver, kidney, heart, brain and muscle homogenates. Incubations of taurine and acetate with tissue homogenates were described in the *Materials and Methods*.

Figure 2.8B

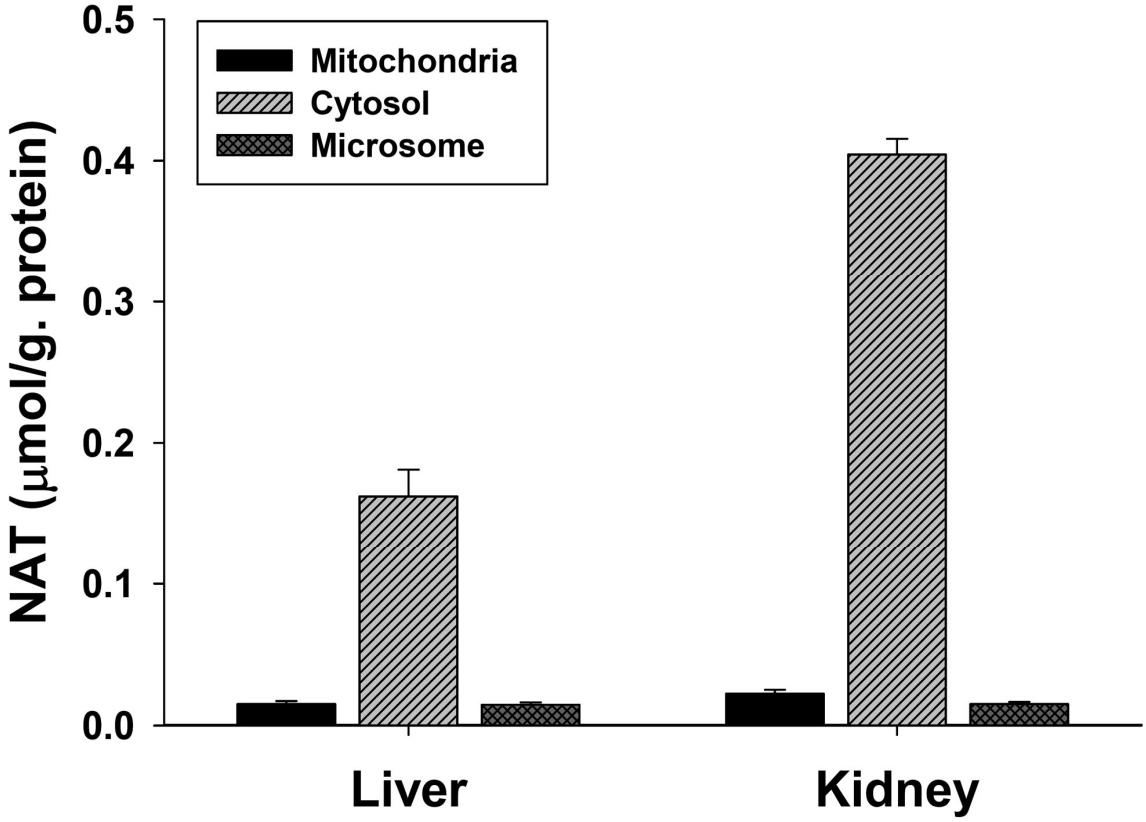

Figure 2.8B NAT biosynthesis by mitochondrial, cytosolic and microsomal factions of mouse liver and kidney. Incubations of taurine and acetate with intracellular fractions were described in the *Materials and Methods*.

Figure 2.9

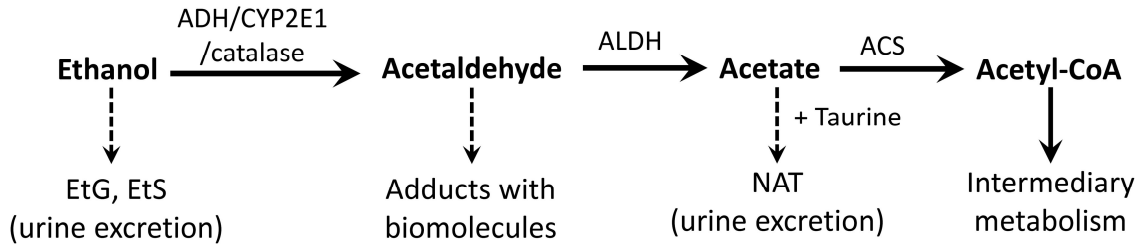

Figure 2.9 The role of NAT and CYP2E1 in ethanol metabolism. Major metabolic pathways of ethanol (arrow with a solid line), encompassing three enzymatic steps and two intermediates, are responsible for converting ethanol into acetyl-CoA, a central metabolite of intermediary metabolism, while minor metabolic pathways (arrow with a dashed-line) affect the toxic effects of ethanol, i.e., the adductions of acetaldehyde with biomolecules contribute to the toxicity, but the formations of EtG, EtS and NAT facilitate the excretion of ethanol and acetate. CYP2E1 contributes to the formation of acetaldehyde, which is further oxidized to acetate, the substrate of NAT biosynthesis.

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