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Hepatic lipid profiling of deer mice fed ethanol using ^1H and ^{31}P NMR spectroscopy: A dose-dependent subchronic study

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Abstract

Chronic alcohol abuse is a 2nd major cause of liver disease resulting in significant morbidity and mortality. Alcoholic liver disease (ALD) is characterized by a wide spectrum of pathologies starting from fat accumulation (steatosis) in early reversible stage to inflammation with or without fibrosis and cirrhosis in later irreversible stages. Previously, we reported significant steatosis in the livers of hepatic alcohol dehydrogenase (ADH)-deficient (ADH^-) vs. hepatic ADH-normal (ADH^+) deer mice fed 4% ethanol daily for 2 months [Bhopale et al., 2006, *Alcohol* 39, 179–188]. However, ADH^- deer mice fed 4% ethanol also showed a significant mortality. Therefore, a dose-dependent study was conducted to understand the mechanism and identify lipid(s) involved in the development of ethanol-induced fatty liver. ADH^- and ADH^+ deer mice fed 1, 2 or 3.5% ethanol daily for 2 months and fatty infiltration in the livers were evaluated by histology and by measuring dry weights of extracted lipids. Lipid metabolomic changes in extracted lipids were determined by proton (^1H) and ^{31}P phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectroscopy. The NMR data was analyzed by hierarchical clustering (HC) and principle component analyses (PCA) for pattern recognition. Extensive vacuolization by histology and significantly increased dry weights of total lipids found only in the livers of ADH^- deer mice fed 3.5% ethanol vs. pair-fed controls suggest a dose-dependent formation of fatty liver in ADH^- deer mouse model. Analysis of NMR data of ADH^- deer mice fed 3.5% ethanol vs. pair-fed controls shows increases for total cholesterol, esterified cholesterol, fatty acid methyl esters (FAMES), triacylglycerides and unsaturation, and decreases for free cholesterol, phospholipids and allylic and diallylic protons. Certain classes of neutral lipids (cholesterol esters, fatty acyl chain ($-\text{COCH}_2-$) and FAMES) were also mildly increased in ADH^- deer mice fed 1 or 2% ethanol. Only small increases were observed for allylic and diallylic protons, FAMES and unsaturations in ADH^+ deer mice fed 3.5% ethanol vs. pair-fed controls. PCA of NMR data showed increased clustering by gradual separation of ethanol-fed ADH^- deer mice groups from their respective pair-fed control groups and corresponding ethanol-fed ADH^+ deer mice groups. Our data indicate that dose of ethanol and hepatic ADH deficiency are two key factors involved in initiation and progression of alcoholic

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fatty liver disease. Further studies on characterization of individual lipid entities and associated metabolic pathways altered in our deer mouse model after different durations of ethanol feeding could be important to delineate mechanism(s) and identify potential biomarker candidate(s) of early stage ALD.

Keywords

Ethanol; Fatty liver; Lipidomics; Alcoholic liver disease; Alcohol dehydrogenase; Deer mice

Introduction

Chronic alcohol abuse is a second major cause of liver disease (NIAAA, 2000; Lieber, 2004; Albano, 2008). Alcoholic liver disease (ALD) causes significant morbidity and mortality among chronic alcohol abusers. The disease is characterized by hepatic steatosis (fatty liver) in early and reversible stage which progresses to inflammation with or without fibrosis and cirrhosis at chronic stage. Although hepatic lipid metabolism is the primary and early target in ethanol-induced liver injury, metabolic basis and mechanism(s) of ALD are poorly understood primarily due to a lack of suitable animal model which mimics human ALD (Hall et al., 2001). Therefore, identification of altered lipid metabolome involved in initiation and progression of ALD could be important for understanding metabolic basis as well as mechanism(s) of ALD and also for identifying its biomarker(s) for early detection and prevention.

Oxidative metabolism of ethanol catalyzed by cytosolic alcohol dehydrogenase (ADH) in the liver is major metabolic pathway for elimination of bulk of ethanol from the body and mostly inhibited during chronic alcohol abuse (Thomas et al., 1982; Nuutinen et al., 1983; Palmer & Jenkins, 1985; Panes et al., 1989, 1993). This is further supported by the studies in laboratory animals and *in vitro* after ethanol exposure (Shore and Theorell, 1966; Baker et al., 1973; Zahlten et al., 1980; Sharkawi, 1984; Kaphalia et al., 1996; Ciucan et al., 2010). On the other hand, hepatic microsomal cytochrome P450 2E1 (CYP2E1) induced after chronic ethanol exposure is advocated as an important pathway for oxidative metabolism of ethanol resulting in oxidative stress due to generation of reactive oxygen species contributing to pathogenesis of ALD (Lieber, 2004; Cederbaum, 2010). In view of relatively very high K_m as compared to that for ADH, role of CYP2E1-mediated oxidation of ethanol and related liver injury are not well understood (Lieber, 2004). Oxidative metabolism of ethanol is coupled to reduction of NAD to NADH leading to significant differences in the NADH/NAD ratio which results in aberrant regulation of metabolism of carbohydrates and lipids, ketogenesis and fatty acid metabolism (Day and Yeaman, 1994). Increased ratio of NADH/NAD is also associated with adipose tissue breakdown and reduced mitochondrial fatty acid β -oxidation, which increase fatty acid pool and subsequently generates triacylglycerides and cholesterol esters (Lieber et al., 1967; Grunnet and Kondrup, 1986; Day and Yeaman, 1994). We also reported that some phospholipids are also altered significantly after ethanol exposure in rat model (Fernando et al., 2010, 2011).

Increased endogenous fatty acids can also facilitate the nonoxidative metabolism of ethanol *via* esterification to fatty acid ethyl esters (FAEEs, catalyzed by FAEE synthase); an alternative mechanism of ethanol disposition during chronic alcohol abuse. In fact, a large concentration of FAEEs has been reported in the plasma and/or pancreatic tissue of laboratory animals after subchronic exposure to ethanol and in alcoholic patients (Kaphalia et al., 2004, 2010; Bhopale et al., 2006, 2011). FAEEs and phosphatidylethanol (PEt) catalyzed by FAEE synthase and phospholipase D, respectively, are classical nonoxidative metabolites of ethanol commonly found during chronic alcohol abuse (Laposata and Lange, 1986; Mueller et al., 1988; Kaphalia et al., 2004). Although biological significance of lipid metabolites of ethanol is not well understood, their *in vivo* and/or *in vitro* hepatocellular toxicity have been reported by us and others (Aydin et al., 2005; Kaphalia and Ansari, 2001; Seitz et al., 2005; Wu et al., 2006). Therefore, a lipid metabolic approach to identify ethanol-induced lipid fingerprint or signature could be important for understanding the mechanism and also to identify biomarker(s) of ALD.

Nuclear magnetic resonance spectroscopy (NMR) is a powerful analytical technique being increasingly used to identify lipid metabolic changes as well as identify various lipid classes involved in fatty liver disease (Fernando et al., 2011). Our previous studies suggest that ADH-deficiency (a metabolic condition prevalent in chronic alcoholics) is an important determinant for ethanol-induced hepatocellular injury and fatty liver (Bhopale et al., 2006; Wu et al., 2006). To understand the role of lipids in pathogenesis of alcoholic fatty liver disease, present study was undertaken to determine dose-dependent lipid metabolomic changes using ^1H and/or ^{31}P NMR spectroscopy in the livers of ADH⁻ and ADH⁺ deer mice fed 1, 2 or 3.5 % ethanol daily for 2 months.

Materials and methods

Chemicals and reagents

Unless specified, all the chemicals, reagents and HPLC and GC grade solvents used in the present study were obtained from Sigma Aldrich Co. (St Louis, MO) or Fisher Scientific (NJ).

Animal experiments

One year old male hepatic ADH negative phenotype (ADH⁻) deer mice, a natural genetic variant of *Peromyscus maniculatus*, and ADH positive (ADH⁺) deer mice obtained from *Peromyscus* Stock Center, University of South Carolina, Columbia, SC, were housed in UTMB's Animal Resource Center. After a mandatory 10 weeks quarantine as per institutional regulation, both strains were divided into two groups, control and experimental. All the animal experiments conducted in this study are in accordance to animal care protocols instituted by UTMB's Institutional Animal Care and Use Committee. Experimental groups were fed Lieber-DeCarli liquid diet (Dyets Inc., Bethlehem, PA) for a week followed by 1, 2 or 3.5% ethanol in the liquid diet for two months (Bhopale et al., 2006; Kaphalia et al., 2010). Controls for both strains were pair-fed with liquid diets containing equivalent calories substituted by maltose-dextrin. At the end of 2 months of ethanol feeding, animals were euthanized by intraperitoneal administration of Nembutal

TM. Livers were excised for gross examination and a portion was fixed in 10% buffered formalin, dehydrated in 70% ethanol and embedded in paraffin blocks for histology and immunohistochemistry. Remaining portions of the livers were stored at -80°C for lipid analysis.

Morphological studies

Standard sections of fixed liver tissue embedded in paraffin were cut (5 µm thick) and stained with Hematoxylin & Eosin (H & E) for the light microscopic examination (Bhopale et al., 2006). Thin liver tissue sections were processed for immunohistochemical detection of oxidative stress and inflammation using antibodies against 4-hydroxynonenal (4-HNE) and CD3, respectively (Fernando et al., 2010; Kaphalia et al., 2010).

Total dry weights of lipids

Liver homogenates were prepared and lipids extracted with methyl tert-butyl ether. The lipid extract was dried under nitrogen and total dry lipid weights determined (Matyash et al., 2008; Fernando et al., 2010).

Lipid metabolomic studies

The lipid extracts were subjected to proton (^1H) and phosphorus (^{31}P) NMR spectroscopy and the NMR data analyzed by pattern recognition analyses as described earlier (Fernando et al., 2010, 2011).

NMR spectroscopy

The dried lipid fractions were dissolved in deuterated chloroform or chloroform/methanol (Sigma-Aldrich, WI) containing tetramethylsilane (TMS) or triethylphosphate (TEP) as internal standard (IS) for ^1H and ^{31}P NMR spectroscopy, respectively. ^1H NMR spectra were obtained using an 800 MHz Varian NMR spectrometer (Varian Inc., CA). ^{31}P NMR analysis was carried out on a 600 MHz Varian Inova NMR spectrometer (Varian Inc., CA) set at 242.69 MHz using a single pulse with proton decoupling only for the lipids extracted from livers of ADH⁻ and ADH⁺ deer mice group fed 3.5% ethanol. Samples were placed in a 5 mm NMR tube and spectra acquired using 5500 scans per sample at 25°C. Identification of phospholipids was accomplished by comparing with standards and spiking the samples with authentic phospholipid standards. The one dimensional spectra were recorded using a single pulse sequence with 256 scans. Spectra were manually phased and the proton signals were referenced to TMS set at $\delta = 0.00$ ppm. The chemical shifts of clearly identifiable peaks were assigned to various metabolites/lipid groups by comparing the published data on lipids as described elsewhere (Chapman and Morrison, 1966; Awl et al., 1989; Sze and Jardetzky, 1990; Fernando et al., 2011).

Pattern recognition analysis

All acquired NMR data were subjected to multivariate statistical analysis. Prior to the analysis, all NMR data were reduced to integral regions of equal width of 0.01 ppm using an 'in house' VNMR macro and the spectral regions between $\delta = 0.2$ to 6.4 ppm (or $\delta = 0.2$ to 2.3 for ^{31}P NMR) and segmented into 620 integrated regions (bin). Baseline corrections

were made using the same macro. The integrated data were further analyzed using the Spotfire Decision Site 9.0 software (TIBCO Spotfire, Somerville, MA). Herein, first, a t-test was performed for all of the integrated regions, and then the regions with statistically significant differences between the two groups ($p = 0.005, 0.01$ or 0.05) were selected for further analysis. These regions were then normalized, and the Z-scores calculated and used for pattern recognition by hierarchical clustering (HC) and principal component analysis (PCA). The % changes between the two groups were calculated by taking the average bin integration value of ethanol-fed and control groups at each given ppm. The analysis was performed among the ethanol-fed vs. control ADH⁻ deer mice, ethanol-fed vs. control ADH⁺ deer mice and ethanol-fed ADH⁻ vs. ADH⁺ deer mice.

Statistical analysis

The data sets were analyzed for statistical significance using Student's t-test and ANOVA or Student-Newman-Keul's multiple comparison test and p value < 0.05 was considered significant. The results are expressed as mean $\pm$ SEM (standard error of mean) of 5 animals per group.

Results

A statistically significant change between the body weights of ethanol-fed group vs. respective control groups were not found at all the three doses of ethanol used in the present study (data not shown). Similarly, no significant oxidative stress and inflammatory response was observed in the liver sections of both strains at all the three doses of ethanol (data not shown). However, extensive pan lobular histological changes (vacuolization) and statistically significant increases for total dry weights of lipids were found only in the livers of ADH⁻ deer mice fed 3.5% ethanol (Fig. 1 and 2). Neutral lipids especially triacylglycerides, cholesterol esters including cholesterol acetate, fatty acyl chain and fatty acid methyl esters (FAMES) were increased in the livers of ADH⁻ deer mice fed 3.5% ethanol (Table 1). Despite no change in total dry lipid weights, a mild lipidomic change was detectable by NMR analysis in ADH⁻ deer mice fed 1 or 2% ethanol (Table 1, Fig. 3, Supplemental Figure 1S). A significant fatty accumulation was not found in ethanol-fed ADH⁺ deer mice groups; a poor separation and loose clustering observed between ADH⁺ deer mice fed 3.5% ethanol vs. respective pair-fed controls is shown in Fig. 4. No appreciable changes for phospholipids were found between ADH⁻ and ADH⁺ deer mice fed 3.5% ethanol by ³¹P NMR (data not shown). With increasing the ethanol dose we found a better clustering and separation of ethanol-fed ADH⁻ deer mice vs. ethanol-fed ADH⁺ deer mice (Fig. 5, Supplemental Figure 1S). Lipid analysis and lipidomic findings presented herein suggest that ethanol dose and hepatic ADH deficiency are two key factors involved in ethanol-induced fatty liver, and most importantly, NMR analysis can detect lipidomic changes even before the onset of overt fatty liver stage. A detailed dose-dependent NMR data analysis to identify lipid metabolomic changes in the livers of ADH⁻ and ADH⁺ deer mice fed ethanol is described below:

¹H NMR data analysis for ethanol-fed ADH⁻ deer mice vs. pair-fed controls

The PCA profiles of lipids obtained for the livers of controls and the ethanol-fed groups at various ethanol doses and related detailed lipidomic changes are summarized in Table 1 and shown in Fig. 3A-C. Overall, PCA profiles show tight clustering and significant separation of ethanol-fed groups from pair-fed controls with increasing the ethanol dose. Integration values were used to define the change between ethanol-fed and control groups and between ADH⁻ and ADH⁺ deer mice fed ethanol. Significant increases were found for total cholesterol (0.68 ppm, resolved), esterified cholesterol (1.02 ppm, resolved) including cholesterol acetate (2.02 ppm), terminal -CH₃ in fatty acyl chain (0.91 ppm), FAMES (3.67 ppm, resolved), triacylglycerides (4.14–4.16, 4.29–4.30 ppm) and overall unsaturation (5.33–5.35 ppm) with decreases for free cholesterol (1.01 ppm, resolved) and allylic (2.04–2.07 ppm) and diallylic (2.83–2.85 ppm) protons in the livers of ADH⁻ deer mice fed 3.5% ethanol. Significant decreases for phosphatidylserine (PS) or phosphatidylinositol (PI) as observed by ¹H NMR could not be resolved by ³¹P NMR in our instrument settings. However, no changes were found for C-1H and C-2H of glycerophospholipid backbone at 1 and 3.5% ethanol doses (Table 1). Increases were found for total cholesterol, free and esterified cholesterol, fatty acyl chains (terminal-CH₃- and -COCH₂-; 2.28–2.32, 2.33 ppm), FAMES, glycerol phospholipid backbone (C-1H and C-2H; 4.08–4.12; 5.22, 5.23 ppm) and, allylic and diallylic protons as compared to decreases for cholesterol acetate, overall saturated acyl C3 chain (-COCHCH₂-) unsaturated fatty acids (-CH=CH-) and glycerol backbones (-C-1H₂, -C-2H₂, -C-3H₂) in ADH⁻ deer mice fed 2% ethanol. Surprisingly, at this ethanol dose, no change for PS/PI was observed. The NMR studies could also detect mild increases for esterified cholesterol, FAMES, fatty acyl chain (-COCH₂CH₂CH₂-), triacylglycerol back bone (C-1H₂ and C-3H₂) and PI/PS in ADH⁻ deer mice fed even 1% ethanol (Table 1). However, these changes were obtained at p > 0.05 with several insignificant bin points. With increasing the ethanol dose, clustering by PCA showed gradual dose-dependent separation of ethanol-fed groups from pair-fed control groups (Fig. 3). Therefore, NMR analysis of lipids as found in ADH⁻ deer mice fed ethanol has advantage over conventional histology and quantitative methods of lipid analysis in terms of sensitivity and specificity. Comparison of the data from ADH⁻ deer mice fed 2% or those fed 3.5% ethanol showed consistent changes among total and esterified cholesterol and FAMES while the other lipid metabolites mentioned above showed not much parallelism between the groups. Overall, lipidomic changes observed in ADH⁻ deer mice fed ethanol are limited to neutral lipids (total and esterified cholesterol and triacylglycerides), and NMR spectroscopy can detect lipidomic changes even before overt fatty liver pathology noticeable by histological and total lipid weight criteria.

¹H NMR data analysis for ethanol-fed ADH⁺ deer mice vs. pair-fed controls

NMR spectral data analysis of lipids from ADH⁺ deer mice fed 1 or 2% ethanol did not show any significant differences in the lipid profiles than those of respective pair-fed controls (data not shown). However, mild changes in the lipid profiles were noticeable in ADH⁺ deer mice fed 3.5% ethanol with increases at lipid groups corresponding to allylic protons (2.05 ppm with 12%), diallylic protons (2.77, 2.78, 2.81–2.86 ppm with 15, 16, 14, 24, 15, 21, 40 and 54%, respectively), CH₃ of FAMES (3.66, 3.67 ppm with 25 and 36%,

respectively) and unsaturation (5.37–5.41 ppm with 14, 23, 30, 27 and 33%, respectively). The PCA profile for ADH⁺ deer mice fed 3.5% ethanol shows five of the control mice and one ethanol-fed mouse were tightly clustered while the remaining five ethanol-fed mice were well separated from the control group and clustered rather loosely (Fig. 4). Therefore the lipidomic changes in ADH⁺ deer mice fed ethanol are not appreciable at the doses used in the present study.

Comparative ¹H NMR data analysis for ethanol-fed ADH⁻ vs. ethanol-fed ADH⁺ deer mice groups

Minor lipid metabolomic changes were observed in ADH⁻ vs. ADH⁺ deer mice fed 1% ethanol (Fig. 5A, Supplemental Figure 1S). At $p = 0.05$, the changes correspond to increases for total cholesterol, esterified cholesterol, fatty acyl chains ($-\text{COCH}_2$) and decreases in allylic and diallylic protons (Table 2). Although the clustering of ADH⁻ group from the ADH⁺ group was evident, it was rather loose indicating mild changes in lipid metabolome. A similar comparison at 2% ethanol dose showed significant changes between the two strains (Table 2; Fig. 5B). In comparison to the 1% ethanol-fed groups, ADH⁻ deer mice fed 2% ethanol were closely clustered as compared to the ADH⁺ deer mice fed 2% ethanol. This includes increases for total cholesterol, esterified cholesterol, cholesterol acetate, fatty acyl chains, unsaturation and triacylglycerides while decreases for the lipid groups corresponding to allylic and diallylic protons, FAMES and phosphatidylcholine (PC) by PCA analysis. Comparison of both strains at 3.5% ethanol dose showed better separation and tighter clustering than those for 1% or 2% ethanol-fed groups (Fig. 5C). The changes associated with the lipid metabolites show increases for total cholesterol, esterified cholesterol, cholesterol acetate, fatty acyl chains, triacylglycerides and unsaturation while decreases were observed in FAME related protons, allylic and diallylic protons, and PC. The lipid classes changed in 2 and 3.5% ethanol fed groups were more or less same. The PCA comparisons of NMR data of lipids from the livers of ADH⁻ vs ADH⁺ deer mice fed 1, 2 or 3.5 % were performed at $p = 0.05$, 0.01 and 0.005 with 16, 61 and 53 bin points, respectively. If we consider $p = 0.05$ for 2 or 3.5% (74 bin points at $p = 0.01$) ethanol doses, this would result in a higher number of bin points (123 and 154 of 620, respectively). We used different cutoffs to keep the most significant bin points to be of interest. Overall, both the NMR data and the lipid weight data for 1% dose suggest no significant change. However, we observed significant change in total cholesterol (0.68 ppm) even with the very restrictive Bonferroni correction value (Table 2) at 2% ethanol dose despite no significant change for total dry lipid weights. As compared to the 2% ethanol dose, number of bin points that were significant with the Bonferroni correction value increased with 3.5% ethanol dose (Table 2). Superimposed ¹H NMR spectra of ADH⁻ and ADH⁺ deer mice fed 3.5% ethanol shows increases for majority of lipid classes in ADH⁻ deer mice group (Supplemental Figure 2S). Overall, dose-dependent comparisons showed increased clustering and increases for total and esterified cholesterol, cholesterol acetate, fatty acyl chains, and decreases for allylic and diallylic protons in 2% or 3.5% ethanol fed ADH⁻ and ADH⁺ deer mice.

Discussion

Alcoholic liver disease (ALD; a major disease among chronic alcohol abusers) encompasses a wide spectrum of clinical and morphological characteristics. Lipid dysregulation (both altered metabolism of lipids and their transport) appears to be the primary event in early stage ALD. We have already published blood alcohol concentrations (BAC) and fatty acid ethyl esters (FAEEs) in the plasma, pancreas and livers of ADH⁻ and ADH⁺ deer mice strains fed ethanol daily for 2 months (Bhopale et al, 2006; Kaphalia et al., 2010). A remarkable increase for BAC (~140 mg%) in ADH⁻ deer mice fed 3.5% ethanol daily for 2 months vs. those in ADH⁺ deer mice fed 3.5% ethanol (~89 mg %) as compared to those (<10 mg%) at 1 or 2% ethanol doses have been reported by us previously (Kaphalia et al., 2010). Therefore, a greater BAC could be attributed to hepatic ADH deficiency in ADH⁻ deer mice fed high ethanol doses. Increases for FAEEs in the blood plasma and pancreas of ADH⁻ deer mice after ethanol exposure have been reported to be parallel to the observed increases for BAC (Kaphalia et al., 2010). Recently, we found that increasing the duration of ethanol exposure from 3 months to 6 months increases in BAC in ADH⁻ deer mice (unpublished data). It is likely that chronic alcohol abuse increases alcohol levels in alcoholics resulting in increased biosynthesis and accumulation of FAEEs. Although a dose-dependent formation of FAEEs in the livers is not reported in this study, correlation of ethanol dose with BAC, FAEEs in the livers and liver injury could explain the mechanism of pathogenesis of alcoholic liver injury resulting in ALD.

It is well documented that CYP 2E1 catalyzed oxidation of ethanol generates hydroxyethyl radical and other reactive metabolites during chronic alcohol abuse (Cederbaum, 2010). In the present study, we found only pan lobular vacuolization/fat degeneration in our model unlike the centrilobular one reported for carbon tetrachloride or acetaminophen (Recknagle et al., 1989; Ito et al., 2006). Therefore, H & E staining and immunohistochemistry results presented herein do not support centrilobular as well as free radical-mediated hepatic toxicity of ethanol in ADH⁻ deer mouse model at the doses and duration of exposure of ethanol used in this study.

Using NMR spectroscopy and histology, we demonstrated dose-dependent lipidomic changes and development of fatty liver, respectively, in ADH⁻ vs. ADH⁺ deer mice fed ethanol. Pattern recognition [hierarchical clustering (HC) and principal component analysis (PCA)] of our NMR data of lipids extracted from livers of ADH⁻ deer mice fed 3.5% ethanol corroborate well with the extent of hepatic vacuolization in H & E stained sections and total dry lipid weight data of livers. However, noticeable NMR-based lipidomic changes in ADH⁻ deer mice fed 1 and 2% ethanol without any changes in total dry lipid weights as found in the present study reiterates application of NMR-based lipidomics in understanding in pathogenesis of ALD. Although we found no significant changes for the dry lipid weights in the livers of ADH⁺ deer mice fed 1, 2 or 3.5% ethanol or ADH⁻ deer mice fed 1 or 2% ethanol in this study, the NMR-based lipidomic changes could be attributed to compositional changes among various lipid classes. An increasing separation and clustering patterns of NMR-based lipidomic data for ADH⁻ deer mice groups vs. pair-fed controls and ethanol-fed ADH⁺ deer mice groups shows a close relationship between the administered ethanol dose and hepatic ADH deficiency.

Increased fatty acid concentrations and their conjugation with other lipids in ethanol-induced fatty liver are reported by us and others (Lieber et al., 1967; Ricchi et al., 2009; Stewart et al., 2009; Fernando et al., 2010, 2011; Niemela and Alatalo, 2010; Loftus et al., 2011). Our present data emphasize the utility of ADH⁻ deer mouse model in understanding the mechanism of ALD. Comparison of our deer mice data with that of rats in many ways is different in terms of ethanol doses, duration of exposure and animal species. Therefore, broad groups rather than individual values were compared in this study to emphasize the importance of a particular class of lipids in understanding fatty liver disease.

Allylic and diallylic protons increased at all time points in our rat model, while there is no consistent direction of change in ADH⁻ deer mice fed ethanol at all doses vs. respective pair-fed controls and a decrease in ADH⁻ vs. ADH⁺ deer mice fed ethanol. In the rat model, the overall unsaturation increased at the early stage of fatty liver while it diminished in the transition stage (Fernando et al., 2010). In addition to increased biosynthesis, dietary uptake and breakdown of adipose tissue, inhibition of peroxisome proliferators-activated receptor (PPAR)- α (a nuclear hormone receptor involved in regulation of fatty acid oxidation and transport) and fatty acid β -oxidation by ethanol exposure can also increase fatty acid pool (Grunnet and Kondrup, 1986; Sozio and Crabb, 2008). Overall increases for cholesterol esters and triacylglycerides as found in the present study could be a result of increased hepatic fatty acids. Therefore, interventional therapy to target the lipid biosynthetic pathways could be a possible therapeutic approach to treat the ethanol-induced fatty liver disease. It is also possible that an increased biosynthesis of triacylglycerides and cholesterol esters as found herein could be a compensatory mechanism to counteract *in situ* accumulation of fatty acids and their cytotoxicity. Alternately, free fatty acids can be disposed as FAEEs *via* nonoxidative pathway in chronic alcohol abusers as reported in previous studies and in the livers of ethanol fed ADH⁻ deer mice (Laposata and Lange, 1986; Kaphalia et al., 2004; Bhopale et al., 2006).

Contrary to the products of classical phase 1 and phase 2 xenobiotic detoxification mechanisms, FAEEs are lipophilic and preferentially accumulate in adipose tissue or lipid-rich organs and cellular membranes of target organs of their formation. Formation of FAEEs in such target organs as livers and their accumulation to sublethal concentrations may cause hepatocellular cytotoxicity resulting into alcoholic liver disease (Laposata and Lange, 1986; Kaphalia and Ansari, 2001; Aydin et al., 2005; Seitz et al., 2005; Wu et al., 2006). The levels of FAEEs increase dose-dependently in plasma and pancreas of ADH⁻ deer mice fed ethanol (Bhopale et al., 2006; Kaphalia et al., 2010). Therefore, studies to determine FAEEs in the livers of these mice could support the role of nonoxidative metabolism of ethanol in ethanol-induced liver injury. Our data show that ethanol feeding increase nonoxidative metabolism of ethanol and develop ethanol-induced fatty liver disease in ADH⁻ deer mouse model. Since endoplasmic reticulum (ER) membrane is a major site for the biosynthesis of cellular lipids, significant lipidomic changes could also be major factor in ethanol-induced ER stress as found only in the livers of ADH⁻ deer mice fed 3.5% ethanol (Kondraganti et al., 2011). Therefore, lipidomic changes including the formation of nonoxidative lipid metabolites of ethanol in our ADH⁻ deer mouse model could be of interest for further investigation regarding mechanism of ALD.

Our NMR-based lipidomic data reported herein stresses the use of ADH⁻ deer mouse model to further investigate the pathways and mechanism(s) of ALD. However, greater levels of cholesterol acetate in ADH⁻ deer mice fed 3.5% ethanol are surprising based upon anticipated prevalence of oxidative metabolism of ethanol in the livers of ADH⁺ deer mice. Contrary to cholesterol acetate data, a consistent increase for protons related to FAMES in ADH⁻ deer mice fed ethanol at all doses indicate that the pathway(s) for FAME formation is induced by ethanol exposure. Therefore, mechanism of side by side conjugation of endogenous methanol and administered ethanol with endogenous fatty acids warrants further investigations and confirmation. Based upon comparisons between the ADH⁻ and ADH⁺ strains fed ethanol and methylation pathway appears to be prevalent in ethanol-fed ADH⁺ deer mice.

It is well known that phosphatidylserine (PS) and phosphatidylinositol (PI) are important phospholipids involved in apoptotic cell death process and signaling events *via* formation diacylglycerol, respectively, in regard to cell death and cell signaling. All proton signals corresponding to phospholipid backbone could not be identified. Our rat model data reflect a decrease in phospholipids specifically for phosphatidylcholine (Fernando et al., 2011). Similar results were also observed in the present study for ADH⁻ vs. ADH⁺ deer fed 3.5% ethanol. NMR spectroscopy can pick very early lipid metabolomic changes and decipher cell death pathways involved in ethanol-induced fatty liver. In summary, ADH⁻ deer mouse model displays dose-dependent fatty changes in the livers after subchronic ethanol exposure, and can have potential for developing chronic model of ALD. Along the line of cluster analysis and PCA of NMR-based lipidomic data, histology and alternative lipid analysis methods can be utilized to study etiopathogenesis and identify biomarker signature of ALD. Undoubtedly, triacylglycerides, total and esterified cholesterol, fatty acyl chains and FAMES are important lipid targets of ethanol exposure, but still there is a need for alternative high throughput approach to identify the individual lipid(s) at various stages of ALD and identify a biomarker signature of ethanol-induced fatty liver for preventive and therapeutic measures. Since NMR spectroscopy can detect lipidomic changes even before the development of overt fatty liver correlating altered lipid metabolome of liver with that of plasma therefore can open new avenues to determine underlying mechanism and identify biomarker(s) of ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Dose-dependent ethanol-induced fatty liver was studied in deer mouse model
- A NMR-based lipidomic approach with histology and dry lipid weights was used
- We used principal component analysis (PCA) to analyze the NMR lipidomic data
- Dose-dependent clustering patterns by PCA were compared among the groups

Fig. 1.

H & E stained liver sections of ADH⁺ (A, C and E) and ADH⁻ (B, D and F) deer mice fed 1, 2 or 3.5 % ethanol, respectively. Dose-dependent vacuolization was observed in the liver sections of ADH⁻ deer mice only as indicated by arrows. (original magnification x 20).

Fig. 2.

Dry weights of lipids extracted from the livers of ADH⁻ and ADH⁺ deer mice fed 1, 2 or 3.5% ethanol daily for 2 months (* - p value 0.05).

Fig 3A

Fig 3B

Fig 3C

Fig. 3.

PCA analysis of ^1H -NMR spectral data of lipids extracted from liver of ADH⁻ deer mice fed 1, 2 or 3.5% ethanol in liquid diet for 2 months (E1-E6, closed squares) and pair-fed control (C1-C6, open squares) mice at $p = 0.01$ (A), 0.01 (B) and 0.005 (C), respectively. The two dimensional plot of the data does not show a clear separation of the control group from the ethanol-fed mice in Fig. 3A, but the separation becomes better as the dose of ethanol is increased. Herein, each point represents a value calculated from an individual spectrum.

Fig. 4.

PCA score plot of ^1H -NMR spectral data of lipids extracted from liver of ADH^+ mice-fed with 3.5% ethanol (E7-E12, closed circles) in liquid diet for two months and pair-fed control (C7-C12, open circles) mice at $p = 0.05$. The data shows the separation of the ethanol-fed mice from that of the pair-fed controls.

Fig 5A

Fig 5B

Fig 5C

Fig. 5.

PCA score plot of ¹H-NMR data of lipid extracted from livers of 1, 2 or 3.5% ethanol-fed (E) ADH⁺ (closed circles) and ADH⁻ (closed squares) mice at p = 0.05 (A), 0.01 (B), and 0.005 (C) for 2 months, respectively. E1-E6 represents the ADH⁻ mice while E7-E12 represents the ADH⁺ deer mice. The 2D plot of data points shows a clear separation of ADH⁻ and ADH⁺ deer mice and the separation becomes more evident at 3.5% ethanol dose.

Table 1

Summary of ^1H NMR chemical shift bins showing altered lipid metabolites in the livers of ADH⁻ deer mice fed 1, 2 or 3.5% ethanol for two months vs. respective pair-fed controls.

Lipid Metabolites	Chemical shift bins (ppm)	1% Ethanol (% Change at p 0.05)	2% Ethanol (% Change at p 0.05)	3.5% Ethanol (% Change at p 0.01)
Cholesterol (total) CH ₃ attached to C13 (C18H ₃) in cholesterol	0.68	ns	+49	+67
Free cholesterol CH ₃ attached to C13 (C18H ₃) in cholesterol	1.01	ns	+48	-18
Esterified Cholesterol CH ₃ attached to C10 (C19H ₃) in cholesterol	1.02	ns	+45	+21
C3 H of esterified cholesterol	4.60 , 4.61–4.62	+45	+39	+177, <u>+204</u> , +180
Cholesterol acetate	2.01–2.02	ns	-17, -16	+50, +51
Terminal CH ₃ group in fatty acyl chain	0.86 , 0.87 , 0.91, 0.92	-24 , -15	+32, +20, +26	+19, +37, <u>+49</u>
CH ₂ in fatty acyl chain C4 and beyond (sat)	1.28–1.31	ns	-11, -12, -17, -14	+21, +16, +29, +43
Fatty acyl chain -COCH ₂ -	2.24 –2.26, 2.27, 2.28, 2.29, 2.31–2.32, 2.33	+26	+58, +70, +61, +55, +49, <u>+34</u> , -34, -34 (2.31, 2.32)	+31, -20 (2.29), +20, +35
Fatty acyl chain -COCH ₂ CH ₂ CH ₂	1.34– 1.37 , 1.38	+14 , +30	ns	+56, +45, +31, +33, +38
CH ₂ in acyl C3 (saturated chain) or COCH ₂ CH ₂	1.61–1.62	ns	-26, -14	+13, +20
-O-CH ₃ in methyl ester	3.67, 3.68	+67	+68	+45
Allylic-CH=CH-CH ₂ -	2.04–2.07	ns	+11, +14, +18, +25	-24, -13, -14, -24
Diallylic =CH-CH ₂ -CH=	2.80–2.83, 2.84, 2.85	ns	+38, +46, +39, +42, +53	-25, -24
CH=CH in fatty acyl chain (unsaturated fatty acid)	5.33–5.35	ns	-15	+46, +49, +29
Triacylglyceride- C-1H ₂ in glycerol backbone	4.14–4.16, 4.17	+45	-44, -52, -57	+17 (p 0.04)
Triacylglyceride- C-3H ₂ in glycerol backbone	4.28 , 4.29–4.30	+71	-58, -60	+93, +67
Triacylglyceride- C-2H in glycerol backbone	5.27	ns	-51	+182
-CH ₂ -O-P- in PS/PI C-3H ₂ in glycerol phospholipid backbone	3.95 –3.97	+97	ns	-49(p 0.04), -48(p 0.03), -45 (p 0.02)
C-1H in glycerol phospholipid backbone	4.08–4.12	ns	+41, +37, +36, +36, +29	ns
C-2H of glycerophospholipid backbone	5.22–5.23	ns	+81, +87	ns

At 1% none of the important ppm's relevant to the lipid metabolites are significant at p 0.01.

At 2% the highlighted changes are significant at P 0.01.

At 3.5% the highlighted changes are significant at P 0.005.

Underlined changes 8.0 e-5 (0.05/620).

Table 2

Summary of ^1H NMR chemical shift bins showing altered lipid metabolites in the livers of ADH^- vs. ADH^+ deer mice fed 1, 2 or 3.5% ethanol for two months.

Lipid Metabolite	Chemical shift bin (ppm)	1% Ethanol (ADH^- E vs. ADH^+ E) % Change at p 0.05	2% Ethanol (ADH^- E vs. ADH^+ E) % Change at p 0.01	3.5% Ethanol (ADH^- E vs. ADH^+ E) % Change at p 0.01
Cholesterol (Total)	0.68	+29	<u>+54</u>	<u>+86</u>
CH_3 attached to C13 (C18H_3) in cholesterol				
Esterified Cholesterol	1.02	+34	+23	<u>+67</u>
CH_3 attached to C10 (C19H_3) in cholesterol				
C3H of esterified cholesterol	4.60–4.62, 4.59–4.62	ns	+120, +139, +127	+111, +198, <u>+225</u> , <u>+215</u>
Cholesterol Acetate (2.03)	2.01–2.02, 2.03	ns	+54, +46, +19	+33, +31
Terminal CH_3 gps in fatty acyl chain	0.87, 0.91, 0.92	+20	+37	<u>+16</u>
CH_2 in fatty acyl chain C4 and beyond (sat)	1.25, 1.26	–17	–20, –11	–24, <u>–21</u>
Fatty acyl chain - $\text{COCH}_2\text{CH}_2\text{CH}_2$	1.28, 1.30–1.33	ns	+21, +27, +31, +25, +28	+16, +21, +17, +20
CH_2 in acyl C3 (saturated chain) or COCH_2CH_2	1.60, 1.61–1.62	ns	+22, +33	+26, +14
Fatty acyl chain - COCH_2 -	2.26–2.28 , 2.31 2.26, 2.27	+30, +38, +17	+55	+64, +53
-O- CH_3 in methyl ester	3.65–3.67, 3.68, 3.69	ns	–52, –56	–25, –32, <u>–47</u>
Allylic- $\text{CH}=\text{CH}-\text{CH}_2$ -	2.05–2.06 , 2.07–2.11, 2.04, 2.05, 2.07	–22, –23	–20, –34, –38, –41, –42	–12, –10 (p 0.04), –16 (p 0.03)
Diallylic $=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$	2.81 , 2.83, 2.85	–23	–23 (p 0.05), –18 (p 0.05)	–18, –21, –20
$\text{CH}=\text{CH}$ in fatty acyl chain (unsaturated fatty acid)	5.35 , 5.34, 5.35	ns	+33	+27, +12
Triglyceride- C1H_2 in glycerol backbone	4.14–4.16	ns	+72, +109, +92	ns
Triglyceride- C3H_2 in glycerol backbone	4.29–4.30	ns	+229, +150	+113, +83 (p 0.03)
Triglyceride- C2H in glycerol backbone	5.26, 5.27	ns	+369	+155 (p 0.03), +153 (p 0.02)
- $\text{N}^+(\text{CH}_3)_3$ in PC	3.34	ns	ns	–66 (p 0.05)
- $\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ in PC	3.83, 3.84	ns	–59	–39 (p 0.05), –47 (p 0.05)
C1H in glycerol phospholipid backbone	4.10–4.11	ns	–28, –27	ns
-P-O- CH_2 -in PC	4.42	ns	–62 (p 0.05)	–72 (p 0.05)

At 3.5% the highlighted changes are significant at $P < 0.005$.

Underlined changes represent ppm's significant using Bonferroni correction $P < 8.0 \times 10^{-5}$ (0.05/620).