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Lipidomic Changes in Rat Liver after Long-Term Exposure to Ethanol

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Abstract

Alcoholic liver disease (ALD) is a serious health problem with significant morbidity and mortality. In this study we examined the progression of ALD along with lipidomic changes in rats fed ethanol for 2 and 3 months to understand the mechanism, and identify possible biomarkers. Male Fischer 344 rats were fed 5% ethanol or caloric equivalent of maltose-dextrin in a Lieber-DeCarli diet. Animals were killed at the end of 2 and 3 months and plasma and livers were collected. Portions of the liver were fixed for histological and immunohistological studies. Plasma and the liver lipids were extracted and analyzed by nuclear magnetic resonance (NMR) spectroscopy. A time dependent fatty infiltration was observed in the livers of ethanol-fed rats. Mild inflammation and oxidative stress were observed in some ethanol-fed rats at 3 months. The multivariate and principal component analysis of proton and phosphorus NMR spectroscopy data of extracted lipid from the plasma and livers showed segregation of ethanol-fed groups from the pair-fed controls. Significant hepatic lipids that were increased by ethanol exposure included fatty acids and triglycerides, whereas phosphatidylcholine (PC) decreased. However, both free fatty acids and PC decreased in the plasma. In liver lipids unsaturation of fatty acyl chains increased, contrary to plasma, where it decreased. Our studies confirm that over-accumulation of lipids in ethanol-induced liver steatosis accompanied by mild inflammation on long duration of ethanol exposure. Identified metabolic profile using NMR lipidomics could be further explored to establish biomarker signatures representing the etiopathogenesis, progression and/or severity of ALD.

Keywords

Ethanol; fatty liver; metabolomics; lipidomics; lipids

Introduction

Alcoholism is a complex addictive disorder affecting millions of individuals around the world. It is a chronic disorder characterized by excessive ingestion of alcohol and associated alcoholic liver disease (ALD) ranging from steatosis, steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (Purohit et al., 2005; Purohit and Brenner, 2006). The earliest liver

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Conflict of Interest Statement

The authors do not have any conflict of interest to disclose.

injury observed in alcoholics is steatosis (fatty liver) which is characterized by large amounts of fat deposited in hepatocytes (Purohit et al., 2009). Alcoholic fatty liver is reversible if the alcoholic abstains from drinking, otherwise it advances to irreversible stages of liver diseases (Moriya et al., 2009). For a long time, alcoholic fatty liver was considered to be a benign condition, but increasing evidence suggests that it sensitizes the liver to pathologic injury (Apte et al., 2005; Diehl, 2002; Purohit et al., 2009).

The severity and prognosis of ethanol mediated liver diseases generally depend on the concentrations of ethanol and its metabolites in the body, and the frequency and the duration of alcohol abuse. The progression of alcoholic steatosis to alcoholic steatohepatitis is a key step in the development of ALD. About 40–60% of patients with alcoholic steatohepatitis (significant fat accumulation together with neutrophil infiltration) are reported to progress to cirrhosis stage even if abstaining from drinking (Diehl 2002; Lucey et al., 2009; Miranda-Mendez et al., 2010). To confirm the degree of damage in the liver, a liver biopsy is required because no noninvasive diagnostic tests are available to distinguish steatosis from steatohepatitis or the fibrosis stage of cirrhosis (Bergheim et al., 2005). The complexity of ALD associated diseases lead to investigate the underlying mechanisms involving different stages and to identify their potential biomarkers (Litten et al., 2010). With the growing problem of obesity, up to 20% of the population is estimated to have fatty liver known as nonalcoholic fatty liver (Browning and Horton, 2004). Nonalcoholic fatty liver also leads to liver disease, generally termed nonalcoholic fatty liver disease (NAFLD). ALD and NAFLD have many similarities. A widely accepted mechanism for both is a two hit model where fat accumulation sensitizes the liver to a second hit leading to inflammation (Browning and Horton, 2004; Day and James, 1998). Previous work from our lab and by others has demonstrated that the lipid accumulation after ethanol consumption involves one or more of the following lipids: fatty acids (free and esterified), triglycerides (TGs), cholesterol (free/esterified) and phospholipids (Fernando et al., 2010). How these changes progress to the next level of ALD (steatohepatitis) are not well defined and needs further investigation.

Metabolomics identifies metabolites with analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry, and the application of statistical and multivariate methods for information extraction and data interpretation. Many compounds can be detected simultaneously, thus allowing the possibility of identifying potential biomarkers for a particular disease (Fernando et al., 2010). The goal of defining biomarkers is to utilize readily available urine or blood for metabolomic profiling that reflects damage (injury) in tissues. We hypothesize that the changes in lipid metabolome of plasma/liver can serve as a biomarker signature to represent the progression of ALD. Understanding the lipid changes are important, as lipids are among the least studied cellular biomolecules, which exist as aggregates within the membranes providing multiple functions. Our results in this paper demonstrate that ethanol consumption for longer periods causes significant intrahepatic fat accumulation with an altered hepatic and plasma lipid metabolome as well as early signs of inflammation (steatohepatitis) and oxidative stress and demonstrates the potential of NMR spectroscopy in the identification of a differential lipid metabolome at various stages of ALD.

Materials and Methods

Animals and treatment

As reported previously, male Fischer 344 rats (~ 6 weeks old) purchased from Harlan (Indianapolis, IN) were housed in a humidity and temperature controlled animal room with automatic 12h light/dark cycles (Fernando et al., 2010). After one week, animals were housed individually in cages and provided with regular Lieber-DeCarli liquid diet (cat. # 710260) [carbohydrates (11% of calories), protein (18%), fat (35%)] and alcohol or

maltrose-dextrin (cat. # 402851 and 36% calories) (Lieber and DeCarli, 1989) (Dyets Inc., Bethlehem, PA). Pair-feeding of animals was done to minimize confounding variables such as weight gain and caloric intake between the pair-fed controls and ethanol-fed experimental groups. The amount of ethanol in the liquid diet was increased gradually from 1% to 5% and then maintained at 5% (Fernando et al., 2010). The diets were prepared and changed daily and weights of animals and daily intake of liquid diet were recorded each week. Groups of animals from the control and ethanol-fed group were euthanized at the end of 2 and 3 months by administration of an intraperitoneal injection of Nembutal (sodium salt, 80 mg/kg). The blood was collected from the heart in heparinized tubes, centrifuged at 1,000 g for 10 minutes and plasma separated and stored at -80°C . Livers were excised, blotted and weighed. Hematoxylin and Eosin (H&E) and immunohistochemistry using 4-hydroxynonenal (4-HNE) and CD3 antibodies were performed (Fernando et al., 2010). Oil Red O staining was done on frozen liver sections. Oxidative stress was further evaluated by measuring malondialdehyde (MDA) in liver homogenates by the Oxiselect TBARS assay kit STA-330 (Cell Biolabs, Inc. CA). The remaining frozen liver was stored at -80°C for biochemical analysis.

Biochemical parameters

Markers of liver injury, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were assayed using respective kits from Biotron Diagnostics Inc. (Hemet, CA). Liver homogenates were prepared and lipids were extracted as described previously with minor modifications (Gyamfi et al., 2008; Zhou et al., 2006). Briefly, frozen liver (250 mg) samples were thawed on ice and homogenized in 2 ml of cold lysis buffer containing 18 mM Tris pH 7.5, 300 mM mannitol, 50 mM EGTA (ethylene glycol-bis (2-aminoethyl ether)-N,N,N',N'-tetra acetic acid, and 0.1 mM PMSF (phenylmethane sulfonyl fluoride). 400 μl of each sample homogenate was transferred to a separate glass tube, 4 ml of chloroform/methanol mixture (2:1 v/v) was added and vortexed. The samples were gently shaken on a rotating shaker overnight, 800 μl of distilled water was added, vortexed, and centrifuged at 3000 g for 5 minutes. The lower phase of the mixture was collected and dried under a stream of nitrogen. Quantitative determination of free fatty acid (FFA), free cholesterol, total cholesterol and TG was done using assay kits purchased from Wako Diagnostics, Wako Chemicals USA Inc., Richmond, VA (kits used are HR series NEFA-HR(2), Cholesterol E, Free cholesterol E and L-TYPE TG H).

Lipid extraction from plasma and livers for NMR spectroscopic analysis

Frozen plasma samples were thawed on ice and lipids were extracted with methyl tert-butyl ether (MTBE) and dried under nitrogen (Fernando et al., 2010; Matyash et al., 2008). Frozen liver (250 mg) was homogenized in methanol and extracted with MTBE in a manner similar to plasma samples (Fernando et al., 2010).

^1H NMR analysis of plasma and liver lipids

The NMR spectra of lipids extracted from plasma and livers were obtained as described previously (Fernando et al., 2010). The dried lipid fractions were dissolved in deuterated chloroform (Sigma-Aldrich, WI) containing tetramethylsilane (TMS) as an internal standard (Beckonert et al., 2007). The final volume of the sample was made up to 750 μl in a 5 mm NMR tube (Wilmad Lab glass, NJ). ^1H NMR spectra were obtained using a 750 or 800 MHz Varian NMR spectrometer (Varian Inc., CA). 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 metabolite/lipid groups by comparing the published data on lipids as described previously (Fernando et al., 2010).

³¹P NMR analysis of liver lipids

The ³¹P NMR analysis of the livers was performed as described previously (Fernando et al., 2010). The dried lipid extracts were dissolved in 0.75 ml deuterated chloroform-methanol (2:1, v/v) containing triethyl phosphate as an internal standard. All experiments were 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. Samples were placed in a 5 mm NMR tube and spectra acquired using 5500 scans per sample at 25°C. Identification of phospholipids was achieved by comparing with standards, and spiking the samples with authentic phospholipids (Fernando et al., 2010).

Pattern Recognition analysis

All acquired NMR data were subjected to multivariate statistical analysis as described previously (Fernando et al., 2010). Prior to the analysis, all NMR data was 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 8.4 or 8.6 ppm for plasma and liver ¹H NMR samples, respectively and segmented into 820 or 860 integrated regions. All ³¹P NMR samples were also binned into 0.01 ppm regions. Base line corrections were also performed 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 ($p \leq 0.01$ or 0.05) were selected for further analysis. These regions were then normalized, and the Z-scores calculated were used for data clustering 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 control and ethanol-fed animals at each ppm.

The ¹H NMR data of plasma and liver lipids was further clustered by taking different combinations: 1 and 2 months, 2 and 3 months and 1 and 3 months. The 1 month data used for comparisons was from our previous work (Fernando et al., 2010). For this analysis process the binning data obtained in the initial analysis was used and analyzed using Spotfire Decision Site 9.0. In every case the data were divided into two groups (control and ethanol-fed) irrespective of the duration of ethanol feeding time at the point of t-test calculation.

Results

Body weights and liver weights

Whole body weights and liver weights marginally increased in ethanol-fed animals, but were not statistically significant (data not shown). Plasma ALT and AST levels increased in alcohol-fed groups at 2 and 3 months than pair-fed controls. The increases were statistically significant only at 2 months. However, a large standard deviation was observed for those fed ethanol for 3 months and thus could not achieve the statistical significance (Table 1).

Liver Histology and Immunohistochemistry

Both ethanol-fed groups showed vacuolization and fatty deposition as confirmed by H & E staining and Oil Red O staining (Figure 1). Further, immunohistochemistry of liver sections from ethanol-fed groups showed no inflammation, as evidenced by lack of staining of CD3 positive cells at 2 months, but showed mild inflammatory infiltrate at 3 months. No apparent oxidative stress was noticed in the livers at 2 months while mild oxidative stress was apparent at 3 months (6/9) by immunohistochemistry using 4-HNE specific antibodies and MDA quantification (Table 1, Figure 1).

Biochemical Changes

Plasma and hepatic TG, cholesterol and nonesterified fatty acids (FFAs) in ethanol-fed and pair-fed control at 2 and 3 months are summarized in Table 1. The average values of plasma total cholesterol, free cholesterol and TG were similar at 2 and 3 months for ethanol-fed group as well as pair-fed controls. However, FFAs were increased at 3 months in both ethanol-fed groups as well as in pair-fed control. Plasma TG increased and FFA decreased significantly at both time points while plasma total cholesterol showed a significant decrease at 3 months in ethanol-fed animals.

As shown in Table 1, except for the average values for hepatic cholesterol (total and free) in the 3 month ethanol-fed rats, all values for the other parameters were similar at 2 and 3 months for both pair-fed control and ethanol-fed groups. The average values of hepatic TGs for ethanol-fed rats were significantly higher at both time points. Similarly, average values of hepatic total/free cholesterol increased at both time points but were statistically significant only at 3 months. However, hepatic FFA increased significantly in the ethanol-fed rats as compared to controls at both time points.

¹H NMR spectral analysis of plasma lipids

The ¹H NMR spectra of plasma lipids of ethanol-fed rats showed marked differences as the time progressed. Hierarchical clustering (HC, data not shown) and principal component analysis (PCA) showed significant differences between 2 months and 3 months data. This observation was made by the number of binning points at a given statistical significance. Pattern recognition using PCA from individual binning data showed distinct clustering patterns after ethanol treatment. The PCA data at 2 and 3 months at $p \leq 0.01$ for ethanol-fed vs. pair-fed control group showed two main clusters (Figure 2A and 2B). In both cases, the clusters predominantly consisted of pair-fed control and ethanol-fed animals with minor exceptions. However, the clustering pattern (distribution of the animals) indicated the effect of long term ethanol exposure. Individual chemical shifts (bin points) showing a differential positive or negative shifts from the pair-fed control group are summarized in Table 2. These differences corresponds to the ethanol induced changes in the plasma lipids. The decreases were observed in the chemical shifts representing cholesterol (free and esterified) and unsaturation only at 3 months, diallylic and free fatty acid related protons at 2 and 3 months, fatty acid methyl esters and phosphatidylcholine (PC) related protons only at 2 months in ethanol-fed group. A clear increase in the TGs was observed at 3 months while protons related to saturated acyl chains increased at 2 and 3 months. The analysis in the proton region of TGs and phospholipids make the assignment difficult due to many overlapping peaks.

¹H NMR spectral analysis of liver lipids

Figure 2C and 2D shows the PCA of the ¹H NMR metabolomic data of liver lipid extracts at $p \leq 0.01$. In figure 2C, the clustering of the ethanol-fed animals was distinct from the pair-fed controls, with a tighter cluster observed for the 2 months ethanol-fed rats. The corresponding pair-fed controls were clustered loosely as compared to the ethanol-fed rats. A clear separation between the control and ethanol-fed groups was observed at 2 months as compared to that at 3 months. In figure 2D, the pair-fed control rats were tightly clustered with 4 of the ethanol-fed animals, and the other ethanol-fed rats even though away from the pair-fed controls, were clustered in a very loose manner. The clustering pattern showing a cluster of 4/9 ethanol-fed rats in the control group cluster show mild fatty liver as compared to the other ethanol-fed rats. Comparison of the clustering patterns at 2 and 3 months show that at 2 months, all ethanol-fed rats are in a similar stage of fatty liver as compared to that at 3 months.

Analysis of liver lipids at 3 months at $p \leq 0.05$ showed increases in protons corresponding to free cholesterol and the effected individual lipid groups are summarized in Table 2. Increases were observed in protons related to allylic, diallylic and fatty acyl chains (2.28 ppm) from free fatty acids, and/or cholesterol acetate at both time points. Protons related to lyso-phosphatidylethanolamine (LPE), phosphatidylserine (PS), lyso-phosphatidylcholine (LPC) and TGs showed an increase at 2 months while protons related to PE showed an increase at both time points. Decreases in protons related to fatty acid methyl esters and PC were observed at both time points in the ethanol-fed groups. Overall, an increase in cholesterol, PE/ PS, TGs and the unsaturation of acyl chains and a decrease in PC and fatty acid methyl esters were observed in ethanol-fed animals.

³¹P NMR spectral analysis of liver lipids

Using ³¹P NMR spectroscopy, we identified the changes in the phospholipids in the livers of ethanol-fed animals at both time points (Figure 3A and 3B). ³¹P NMR data is useful as it only identifies changes in phospholipids and further confirms our ¹H NMR data. The clustering pattern shows a separation between the pair-fed controls and ethanol-fed animals indicating changes in phospholipids due to ethanol feeding. The patterns observed at 2 and 3 months show a different distribution of points indicating the different response of the phospholipids upon long term exposure of ethanol. The major phospholipids, observed are PC, PE, LPC and LPE. PC, showed a decrease at both time points in ethanol-fed animals. Peaks arising from other head groups such as PS, phosphatidylinositol (PI) and sphingomyelin (SM) appeared to be minor in some animals (Fernando et al., 2010). The effected individual phospholipids are listed in Table 3.

Combined analysis of ¹H NMR data of plasma and ¹H and ³¹P NMR data of liver

We further clustered the data based on the different time points for plasma ¹H-, liver ¹H-, and liver ³¹P NMR in all possible combinations (1, 2 and 3 months). For this purpose we used our previously published one month data (Fernando et al., 2010). Comparison of the combination of data was important as from the individual time points (1, 2, and 3 months) we observe that the clustering pattern of the animals varied depending on the duration of ethanol exposure. Figure 4 shows the PCA of plasma lipids obtained using ¹H NMR. PCA of 1 and 2 months (Figure 4A) reveals a clear separation with minor exceptions of the two groups after ethanol feeding and a clear separation among the 2 month pair-fed control and ethanol-fed animals. In the same figure, two of the ethanol-fed rats at 1 month were clustered with the 1 month pair-fed control rats. Overall, this figure shows the lipid plasma profiles are different with long term ethanol exposure. The PCA of the 2 and 3 months data (Figure 4B) reveals a clear separation among the time points as well as the treatment groups, but the separation among the 3 month pair-fed control and the ethanol group is now less. Figure 4C shows the PCA obtained for 1 and 3 months. In figure 4C all four groups were well separated but with different degree of tightness in the clustering pattern. Tighter clustering was observed for the 1 and 3 month pair-fed controls and the 1 month ethanol-fed rats. However, the 3 month ethanol-fed animals were clustered rather loosely. In all comparisons, increases were observed in fatty acyl chains and decreases in diallylic protons and -CH₂CO- in fatty acyl chains. Many changes in the plasma lipids were progressive from 1 to 2 and 2 to 3 months, but when comparing 1 to 3 months the changes were not additive. Figure 5 and Figure 6 show the clustering of liver ¹H and ³¹P NMR data by PCA at different time points. Clustering of these data using PCA shows different degree of separations. In Figure 5A, complete separation was observed among the pair-fed control and the ethanol-fed groups. Herein, the separation among the ethanol-fed 1 and 2 month rats were also distinct. However, among the pair-fed controls the separation was clear with some overlapping between the 1 and 2 months. Combined PCA of 2 and 3 months data (Figure 5B) and 1 and 3 months data (Figure 5C) were not distinctly separated as the 1 and 2 month liver lipids.

Analysis of liver lipid ^1H NMR data showed consistent changes from 1 to 2 and 2 to 3 months with increases in cholesterol, allylic and diallylic protons, and $-\text{CH}_2\text{CO}-$ in fatty acyl chains. A similar analysis of the liver lipid ^{31}P NMR data using PCA was carried out and the results are shown in Figure 6 (A, B and C). In these plots, the ethanol-fed animals were well separated from the pair-fed controls with minor exceptions, however, the separation among the time points were not as distinct.

Discussion

The pharmacological/pathological effects of alcohol depend on the genetic makeup, dosage, levels of ethanol metabolites in the body, duration of ethanol exposure and nutritional status (Purohit et al., 2005; Purohit and Brenner, 2006). In our previous study, we evaluated early lipidomic changes associated with ethanol exposure in rats (Fernando et al., 2010). In the present study, we evaluated liver injury and lipidomic changes in rats fed for a longer period (2 and 3 months) to get a better understanding of the mechanism underlying the development of fatty liver and its progression to steatohepatitis. We did observe signs of inflammation and mild oxidative stress in livers of ethanol-fed animals at 3 months suggesting the initial development of steatohepatitis. Such histological changes are consistent with the widely accepted two hit model for the development of ALD (Browning and Horton, 2004; Day and James 1998). Another indicator of early liver injury observed was a marginal increase in plasma ALT and AST in ethanol-fed animals at 3 months.

The analysis of NMR data of plasma lipids shows that, the total cholesterol levels decreased only at 3 months. The liver lipids showed an increase in total cholesterol at 2 months, with free cholesterol increasing at the chronic stage and were supported by a significant increase observed in the hepatic total and free cholesterol at 3 months by biochemical assays. The observed increase in hepatic free cholesterol levels at the latter stage is similar to that described during the progression of NAFLD to NASH (Puri et al., 2007).

Circulating TGs have been positively correlated with acute and chronic ethanol toxicity in experimental animals and humans, possibly due to the decreased clearance of TGs from blood and/or decreased oxidation of fatty acids (Barson et al., 2009). However, identification of protons related to TGs was not clearly apparent by NMR data due to its overlapping signals with those of the glycerol backbone and different groups of phospholipids. Increased TGs in the plasma at 3 months and hepatic TGs at 1 month (Fernando et al., 2010) and at 2 months is consistent with the data obtained by biochemical analysis. However, the increases observed in the plasma TGs at 2 months and the hepatic TGs at 3 months by biochemical assays were not clearly identifiable through NMR due to overlapping peaks. Increased hepatic TGs block the TG synthesis leading to an increase in free fatty acids (FFAs). This oversupply of FFAs causes hepatic damage (Alkhoury et al., 2009). TG accumulation itself in the liver may not be harmful to hepatocytes as it may protect against FFA-induced lipotoxicity (McClain et al., 2007).

FFAs are known to contribute to fatty liver in ALD and/or NAFLD (Alkhoury et al., 2009; Cheung and Sanyal, 2008; Donnelly et al., 2005). Fatty acids are part of building blocks of phospholipids. Ethanol stimulates hepatic fatty acid synthesis (Weltman et al., 1998). The oxidation of alcohol to acetaldehyde concomitantly reduces NAD to NADH. The increased NADH promotes fatty acid synthesis and opposes lipid catabolism leading to fat accumulation in hepatocytes (Galli et al., 1999; Lieber and Schmid, 1961; Lieber et al., 1966). In NAFLD, FFAs are the major source of fat accumulation in the liver (Baker et al., 2010). This may be a result of de novo lipogenesis, dietary fatty acids, and/or insufficient fatty acid utilization (Baker et al., 2010). Hepatic lipid loading appears to be mainly determined by the availability of FFA's which are unaltered in NAFLD and NASH

(Donnelly et al., 2005; Puri et al., 2007). Our plasma lipid NMR analysis, similar to biochemical data, showed a decrease in the peak corresponding to $-\text{CH}_2\text{CO}-$ protons (2.26–2.30 ppm) while the hepatic lipid analysis showed an increase at both time points. In summary, changes we observed in FFAs by NMR suggest the involvement of FFAs in the development of ethanol induced fatty liver and monitoring the NMR peaks relevant to FFA in the metabolite analysis could be utilized as a method for identifying ethanol-induced fatty liver and/or its progression.

Fatty acid methyl esters are present in low amounts in the plasma and tissues and can be formed either by transfer of a methyl group from S-adenosyl methionine (SAM) to a free fatty acid (Akamatsu and Law, 1970; Zatz et al., 1981) or by fatty acid ethyl ester synthase mediated conjugation of free fatty acids with endogenous methanol (Kaphalia et al., 1995, 1999). Decreased protons related to $-\text{OCH}_3$ (3.64–3.66 and 3.67–3.70 ppm) in the plasma lipids at 2 months (Table 2) and a similar decrease in the hepatic lipids at 2 and 3 months (Table 3) shows possible involvement of SAM-mediated mechanism in the development of fatty liver. However, if this decrease is due to decreases in SAM and/or impaired methyl transferases remain to be determined.

SAM is also involved in the synthesis of PC from PE (Reo et al., 2002). Previously, we showed a decrease in the PC levels at 1 month of ethanol feeding (Fernando et al., 2010). In the present study, this decrease was further confirmed by the observed decreases in the plasma lipids at 2 months, and hepatic lipids at 2 and 3 months (3.88–3.94 ppm). Increases in the PE related protons in hepatic lipids at 2 and 3 months provide further support for the involvement of a SAM mediated methylation in the liver. Besides being a structural component, PC serves as a reservoir for lipid second messengers and is involved in the synthesis of other phospholipids (van Meer et al., 2008).

We observed some changes in lysophospholipids which have been shown to be extra cellular signaling molecules associated with a vast array of biological functions including cell survival and apoptosis (Zhao et al., 2011). Lysophospholipids are intertwined through complex metabolic pathways involving fatty acids which serve as the building blocks for phospholipids (Dennis et al., 2010). Changes in membrane phospholipid composition during the progression of steatosis to steatohepatitis may aggravate hepatic insulin resistance and lipid overload by altering functions such as tyrosine kinase signaling or uptake of long chain fatty acids into liver cells (Anderson and Borlak, 2008). Lipids are present in the form of lipid droplets, and little information is known about the role of lipid droplets in the protection of cells by disposing toxic lipids such as nonesterified fatty acids and lysoPC (Anderson and Borlak, 2008).

Stress by the flux of FFAs and their ratio of monounsaturated fatty acids (MUFA)/saturated fatty acids (SFA) are suggested to determine the hepatic damage (Li et al., 2009). We observed an increased saturation of fatty acyl chains in plasma lipids with a concomitant decrease in unsaturation. However, in the liver an increase in mono and polyunsaturation was observed which may contribute to the development of steatosis by enhancing lipogenic lipid metabolism and production of inflammatory molecules (Anderson and Borlak, 2008). PUFAs are linked to sterol regulatory binding protein (SREBP-1) and peroxisome proliferator-activated receptor α (PPAR- α) (a nuclear receptor and transcription factor) and have the ability to redirect glucose into glycogen storage and fatty acids from TG storage into lipid oxidation. Apart from PUFAs, MUFAs also prevent palmitate induced lipoapoptosis by channeling excess saturated fatty acids towards TG synthesis and lipid storage away from activation of lipotoxic cell death. The changes in the liver monounsaturations which we observe here could be an adaptive response to protect the liver,

a concept which needs to be confirmed by identifying the source of unsaturated FFAs in the liver.

Our model consists of ethanol and a high fat diet which itself can cause some lipid deposition when given for a longer duration. However, clinical evidence has shown a significantly elevated ethanol intake in subjects consuming high fat (Kesse et al., 2001). Evidence also suggests greater fat accumulation in ethanol drinkers compared with nondrinkers or in high ethanol compared with low ethanol drinkers (Gruchow et al., 1985; Swinburn et al., 1998). Hence the changes observed here most likely would reflect the changes associated with ethanol consumption. A limitation of our experiments is that at 3 months, pair-fed controls have some evidence of mild NAFLD. A recent report suggests that alcohol may contribute to the development of NAFLD (Baker et al., 2010). The generation of alcohol may come from the conversion of maltose-dextrin to alcohol by gut bacteria in the control diet. Despite the similarities of NAFLD/NASH and steatosis/steatohepatitis, our studies provide an evidence of steatosis and early stage steatohepatitis. Increased free cholesterol, lyso PC, SM, TGs with decreased PC observed in our studies are consistent with published results of NASH and here we demonstrate the use of NMR to obtain a lipid signature reflecting fatty liver (Puri et al., 2007).

Lipidomic approaches provide a broader integrated picture where the underlying mechanism needs to be explored (Dennis et al., 2010). In the search for biomarkers for ethanol induced fatty liver if a single analyte is used as a biomarker it is likely to have a cut off value which leads to false positives. Another common problem is that most traditional alcohol biomarkers are elevated in liver damage by NAFLD (Freeman and Vrana, 2010). Thus, several biomolecules in combination are likely to serve as best biomarkers (biomarker signature) in terms of sensitivity and specificity (Ray et al., 2007; Spira et al., 2007). The underlying reasoning is that one biomarker may not accurately diagnose alcohol abuse and associated tissue injury. A recent report suggests that lipid alterations induced by alcohol are highly tissue specific (Zhao et al., 2011).

Changes observed in cholesterol (free/esterified), fatty acids, TGs, phospholipids specifically PC, and unsaturation of fatty acyl chains in the plasma/livers and their interrelationship with biochemical changes and liver injury markers after short and long term ethanol exposure could have diagnostic value to differentiate ethanol-induced steatosis vs. steatohepatitis and may be advanced stages of the disease (fibrosis and cirrhosis). Therefore, plasma and liver lipidome data sets at various stages of ALD in animal models could be utilized to identify progressive ALD and eventually used as tools to screen liver disease in alcoholic population. The data sets of plasma/liver lipids clustered for different stages of ALD (tighter clustering vs. loose clustering) can also be of predictive value. The altered lipid metabolites at each stage of ALD can be explored for their contributions in progression of ALD.

In summary, time dependent fatty infiltration, inflammation and oxidative stress observed upon ethanol feeding in our animal model can be advanced to inflammatory, fibrosis and cirrhosis stages after longer durations of ethanol exposure. Robust difference between the lipidomic profiles of plasma and livers as revealed by PCA in ethanol-fed animals could be utilized to identify various lipids as biomarkers. The major lipid classes, cholesterol, phospholipids, TGs and overall unsaturation, altered in the alcohol-fed animals as supported by biochemical changes can be utilized to build correlations. Changes in various lipids moieties (FFA, FAME, PC, TGs and Cholesterol) in the plasma and livers can be explored to differentiate various stages of ALD. Therefore, lipid metabolic profiling presented in this study (metabolic panel of diagnostic chemical shifts and patterns) can give a better understanding of pathogenesis of ethanol mediated liver injury. Pattern recognition of

plasma lipids can be used as a non invasive diagnostic tool for ethanol-induced liver damage in a clinical setting.

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Figure 1.

Histology and immunohistochemistry of representative liver sections of rats fed 5% ethanol in liquid diet daily for 2 (AR2) and 3 (AR3) months. Upper left shows H&E stained representative liver sections from the pair-fed controls and ethanol-fed animals with significant fatty deposition, confirmed by Oil Red O staining (lower left). CD3 stained liver sections for pair-fed controls and ethanol-fed animals (upper right) and immuno staining for 4-HNE in pair-fed controls and ethanol-fed animals are shown in the lower right.

Figure 2.

The upper panel represents the PCA of the ^1H NMR data from plasma lipid extracts at 2 (A) and 3 (B) months at $p \leq 0.01$. C7-C19 represents pair-fed control rats and E8-E22 represents ethanol-fed rats. Each point represents a value calculated from an individual spectrum. Filled squares (green) and circles (red) represent pair-fed control and ethanol-fed animals respectively. The lower panels represent PCA of the ^1H NMR data from liver lipid extracts at 2 (C) and 3 (D) months at $p \leq 0.01$. Numbering of pair-fed control (green) and ethanol-fed (red) animals is the same as (A) and (B). The two dimensional plot of the data points demonstrates separation of ethanol-fed rats.

Figure 3.

PCA analysis of ^{31}P NMR data of lipids extracted from liver of pair-fed control and ethanol-fed animals at 2 (A) and 3 months (B) at $p \leq 0.01$. C7-C19 represents the pair-fed controls and E8-E22 represents the ethanol-fed animals. Each point represents a value calculated from an individual spectrum with filled squares and circles representing pair-fed control (green) and ethanol-fed (red) animals, respectively.

Figure 4.

PCA score plots of ¹H NMR plasma lipids. Comparison at 1 and 2 months at $p \leq 0.05$ (A). Comparison at 2 and 3 months at $p \leq 0.01$ (B). Comparison at 1 and 3 months at $p \leq 0.05$ (C). C1-C19 represents the pair-fed controls and E1-E22 represents the ethanol-fed animals. Each point represents a value calculated from an individual spectrum with filled squares (1 month), triangles (2 months) and circles (3 months) representing pair fed control (green) and ethanol-fed (red) animals, respectively.

Figure 5.

PCA score plots of ^1H NMR liver lipids. Comparison at 1 and 2 months at $p \leq 0.05$ (A). Comparison at 2 and 3 months at $p \leq 0.01$ (B). Comparison of 1 and 3 months at $p \leq 0.05$ (C). The inset in (C) represents the comparison of 1 and 3 without the E14, and E20. The numbering and the symbols are as the same as in Figure 4.

Figure 6.

PCA score plots of ^{31}P NMR liver lipids. Comparison at 1 and 2 months at $p \leq 0.05$ (A). Comparison of 2 and 3 months at $p \leq 0.05$ (B). Comparison at 1 and 3 months at $p \leq 0.05$ (C). The numbering and the symbols are as the same as in Figure 4.

Table 1

Biochemical changes representing liver function test, lipid and oxidized lipid contents of the plasma and liver.

	Parameter	Control (AR2)	Ethanol-fed (AR2)	Control (AR3)	Ethanol-fed (AR3)
Plasma	ALT (U/L)	43.25 ± 5.81	59.45 ± 8.99*	43.96 ± 9.39	56.85 ± 14.26
	AST (U/L)	70.32 ± 12.47	99.39 ± 12.95*	68.06 ± 8.52	92.27 ± 25.92
	TG (mg/dL)	127.18 ± 50.44	292.52 ± 117.90*	113.78 ± 18.66	224.89 ± 88.75*
	FFA (mEq/L)	0.25 ± 0.12	0.13 ± 0.11*	0.52 ± 0.14	0.31 ± 0.11*
Liver	TC (mg/dL)	76.31 ± 13.41	65.95 ± 4.21	81.60 ± 6.57	63.87 ± 8.12*
	FC (mg/dL)	20.14 ± 3.87	39.10 ± 9.95*	25.70 ± 2.89	34.64 ± 12.94
	MDA (nmol/mg)	12.76 ± 1.63	12.09 ± 0.62	13.25 ± 2.68	13.30 ± 0.94
	TG (mg/g)	20.69 ± 6.16	39.84 ± 15.33*	18.21 ± 4.52	42.46 ± 13.41*
	FFA (μmol/g)	20.10 ± 2.89	34.66 ± 16.31*	16.59 ± 5.90	31.90 ± 12.90*
	TC (mg/g)	36.65 ± 4.24	56.11 ± 21.94	31.86 ± 6.00	78.41 ± 27.50*
	FC (mg/g)	29.49 ± 5.63	38.86 ± 8.65	26.10 ± 4.98	58.20 ± 21.12*

The values represent the average of 6–9 animals. The details are listed under the materials and methods section. AR2 and AR3 represent data for 2 and 3 months, respectively. Mean ± S D was calculated using the GraphPad InStat 3 program with the Mann-Whitney test.

* Significant compared to control group ($p \leq 0.05$). TG-triglyceride; FFA-free fatty acid; TC-total cholesterol; FC-free cholesterol.

Table 2Significant metabolic differences in the plasma lipids of ethanol-fed rats obtained from ^1H NMR at $p \leq 0.05$.

Chemical Shift (ppm)	Lipid Metabolite	2 months (% Change)	3 months %Change
0.68,0.69	CH_3 attached to C13 (C_{18}H_3) in cholesterol	NS	-30, -40
0.91–0.94	CH_3 attached to C21 (C_{20}H_3) in cholesterol (terminal)	NS	-17, -25, -23, -15
0.99–1.03	CH_3 attached to C10 (C_{19}H_3) in cholesterol (free 1.01, esterified 1.02)	NS	-24, -28, -26, -31, -29
1.06,1.16	Multiple cholesterol protons	NS	-24, -25, -26, -28
1.45,1.46	Cholesterol	NS	-10, -11
1.50–1.58	Cholesterol /fatty acyl chain $-\text{CO}-\text{CH}_2\text{CH}_2-$	NS	-14, -14, -16, -12, -12, -12, -15, -17
2.28,2.29, 2.30	Cholesterol C4 $-\text{CH}_2\text{COOH}-$ in fatty acyl chain (2.28)/ $-\text{CH}_2\text{CO}-$ in fatty acyl chain	-24	-17, -17
3.44–3.50	C3 H of free cholesterol	-18, -20, -22, -24, -26, -29, -30, -29, -27, -25, -25, -25	NS
4.61–4.63	C3 H of esterified cholesterol	NS	-25, -27, -27
0.86,0.87,0.89–0.91	Terminal CH_3 groups in fatty acyl chain	+7, +11, +11	-21 -15
1.28–1.35,1.27–1.35	CH_2 in fatty acyl chain (C4 and beyond saturated)	+17, +18, +15, +15, +16, +13, +12, +10	+15, +20, +19, +28, +28, +25, +21, +15, +11
1.39	$-\text{CO}-\text{CH}_2\text{CH}_2\text{CH}_2-$ in fatty acyl chain	+12	NS
1.61–1.65,1.60–.63	CH_2 in acyl C3 (saturated chain) or COCH_2CH_2	+25, 26, +27, +31, +36	+20, +36, +36, +25
2.28,2.29, 2.30	Cholesterol C4 $-\text{CH}_2\text{COOH}-$ in fatty acyl chain (2.28)/ $-\text{CH}_2\text{CO}-$ in fatty acyl chain	-24	-17, -17
2.80–2.92,2.80–2.85	Diallylic $=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$	-24, -20, -13, -15, -19, -16, -17, -24, -27, -27, -24, -27, -27, -27, -28, -28	-13, -18, -19, -16, -17, -14
3.67–3.70	CH_3 of COOCH_3 in esterified fatty acid/ $\text{O}-\text{CH}_3$ aliphatic methyl ester	-27, -29, -28, -29	NS
5.37–5.41	$\text{CH}=\text{CH}$ in fatty acyl chain (unsaturated fatty acid)	NS	-13, -15, -13, -12, -10
3.32,3.33	$-\text{N}^+(\text{CH}_3)_3$ of PC/choline/sphingomyelin	-25, -20	NS
3.57–3.62	Ph-glycerol	-25, -27, -28, -29, -29, -29	NS
3.88–3.91	$-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ of PC	-30, -25, -21, -25	NS
3.97	$-\text{C}-3\text{H}_2-\text{O}-\text{P}$ in glycerophospholipid backbone	NS	-21
4.09–4.11	$\text{C}-1\text{H}_2$ in glycerol backbone of PL	-21, -22, -23	NS
4.14–4.16	$\text{C}-1\text{H}_2$ in glycerol backbone (TG)	NS	+41, +52, +54
4.21	$-\text{P}-\text{O}-\text{CH}_2-$ in PC head group	-32	NS
4.28–4.30	$\text{C}-3\text{H}_2$ in glycerol backbone of TGs	NS	+74, +62, +59
4.34–4.46	Glycerol backbone of PL / $-\text{P}-\text{O}-\text{CH}_2-$ in PC	-32, -31, -30, -28, -22, -25, -28, -32, -32, -32, -31, -31, -29, -25	NS

Chemical Shift (ppm)	Lipid Metabolite	2 months (% Change)	3 months %Change
5.22	C-2H of glycerophospholipid backbone (PC)	NS	<i>-31</i>
5.26–5.28	C-2H in glycerol backbone of TGs	NS	<i>+62, +61, +51</i>

NS-not significant; 3 month changes shown in italics

Table 3Significant metabolic differences in the liver lipids of ethanol-fed rats obtained from ^1H NMR at $p \leq 0.05$.

Chemical Shift (ppm)	Lipid Metabolite	2 months (% Change)	3 months %Change
0.68	CH_3 attached to C13 (C_{18}H_3) in cholesterol	+33	<i>NS</i>
0.98	Cholesterol (C_{14} free)	NS	+30
1.01	CH_3 attached to C10 (C_{19}H_3) in free cholesterol	NS	+20
1.08,1.09	Multiple cholesterol protons	NS	+30, +30
1.10–1.18	Multiple cholesterol protons	NS	+30, +43, +52, +50, +52, +57, +36, +49, +53
2.26,2.27,2.28	Cholesterol C_4 /- CH_2COOH - in fatty acyl chain (2.28)	+19, +35	+21
2.04–2.06, 2.05,2.06	Allylic- $\text{CH}=\text{CH}-\text{CH}_2$ -/Cholesterol acetate(2.03)	+30, +33, +21	+21, +18
2.14–2.19	- CH_2COOR acyl C2	NS	-40, -51, -50, -62, -65, -62
2.26,2.27,2.28	Cholesterol C_4 /- CH_2COOH - in fatty acyl chain (2.28)	+19, +35	+21
2.37–2.39	$\text{RCH}_2\text{CH}_2\text{CO}$ -		-37, -39, -25
2.76–2.79 2.78,2.81–2.83	Diallylic $=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$	+46, +46, +38, +20	+31, +19, +13, +16
3.67	- CH_2 -O-H in alcohol	+11	<i>NS</i>
3.70,3.71, 3.80	-O- CH_3 methyl ester	-75, -71	-72
5.32,5.33,5.34	$\text{CH}=\text{CH}$ in fatty acyl chain (unsaturated fatty acid)	+36, +32, +29	<i>NS</i>
		NS	<i>NS</i>
3.20,3.21	N- CH_2 -of PE	+60, +15	+74
3.26–3.31	N- CH_2 -of glycerol PE	+55, +72, +76, +84, +107, +167,	<i>NS</i>
3.33	- $\text{N}^+(\text{CH}_3)_3$ of LPC	+383	<i>NS</i>
3.35	- $\text{N}^+(\text{CH}_3)_3$ of PC/choline	-86	<i>NS</i>
3.62	- CH_2 -OH of glycerol	-67	<i>NS</i>
3.81,3.82	C_3H_2 of LPC	+308, +220	<i>NS</i>
3.85–3.87,3.88	- CH_2 - $\text{N}^+(\text{CH}_3)_3$ of PC	-98, -100, -98	-64, -63, -59, -46
3.90,3.91	-C-3H in glycerophospholipid (PC)	-77, -79	<i>NS</i>
3.96–3.98	- CH_2 -O-P in PE/LPE and/or POCH in PS (PL: PE/Pet/PS/PI)	+109, +141, +67	<i>NS</i>
4.0–4.02,4.04–4.05	C_1H of glycerophospholipid (PC)	-64, -79, -87, -83, -72	-40
4.12,4.13	C_1H of glycerol backbone (TGs)	+43, +18	<i>NS</i>
4.23,4.24	- CH_2 -O-CO-R 1,2 diacyl glycerol	NS	-84, -67, -58, -94
4.36	$\text{C}'_1\text{H}$ (- CH_2 -O-P) of glycerophospholipid (choline)	NS	-68
4.37, 4.38	C_3H_2 of glycerol backbone of TGs	+95, +50	<i>NS</i>
5.23,5.24	C_2H of glycerol backbone of PL (PC)	-35, -27	<i>NS</i>

NS-not significant; 3 month changes shown in italics

Table 4

Significant metabolic differences in the liver lipids of ethanol-fed rats obtained from ^{31}P NMR at $p \leq 0.05$.

Chemical Shift (ppm)	Lipid Metabolite	2 months (% Change)	3 months %Change
0.56–0.60 0.52– 60	Phosphatidylcholine	–33, –34, –30, –28, –30	<i>–34, –65, –79, –91, –121, –135, –142, –144, –175</i>
1.26–1.28	Lyso-phosphatidylcholine	+22, +44, +53, +59, +93, +96, +118	NS
1.56–1.69	Phosphatidylserine/Phosphatidylethanolamine	+127, +129, +94, +91, +112, +118, +98	NS
1.93–1.96, 1.91–1.93	Lyso-phosphatidylethanolamine	+79, +105, +163, +248	<i>–41, –47, –49</i>

NS-not significant; 3 month changes shown in italics