Altered Hepatic Lipid Metabolism in C57BL/6 Mice Fed Alcohol: A Targeted Lipidomic and Gene Expression Study

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Running title: Altered hepatic lipid metabolism following alcohol feeding
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List of Abbreviations: ALD: Alcoholic liver disease; FA: Fatty acid; LC/MS/MS: liquid chromatography tandem mass spectrometry; FFA: free fatty acid; FA-CoA: fatty acyl-CoA; FAEE: fatty acid ethyl ester; SO-1P: sphingosine-1-phosphate; SA-1P: sphinganine-1-phosphate; AEA: anandamide; 2-AG: 2-arachidonoylglycerol; BAC, blood alcohol content; ALT, alanine aminotransferase; UPLC, ultra performance liquid chromatography; ESI-MS, electrospray ionizing mass spectrometry; TEA, triethylamine; MRM, multiple reactions monitoring; SIR, selected ion recording; PPAR, peroxisome proliferator-activated receptor; and VLDL, very low density lipoprotein.

Financial Support: The authors gratefully wish to acknowledge the support of grants RC2 AA019413, R01 DK68437, and R01 DK079221 from the National Institutes of Health. Additionally, support from the Columbia University, Clinical and Translational Science Award, Irving Institute, and grant UL1 RR024156 is acknowledged.
Abstract

Chronic alcohol consumption is associated with fatty liver disease in mammals. The object of this study was to gain an understanding of dysregulated lipid metabolism in alcohol-fed C57BL/6 mice using a targeted lipidomic approach. Liquid chromatography tandem mass spectrometry was used to analyze several lipid classes, including free fatty acids, fatty acyl-CoAs, fatty acid ethyl esters, sphingolipids, ceramides, and endocannabinoids, in plasma and liver samples from control and alcohol-fed mice. The interpretation of lipidomic data was augmented by gene expression analyses for important metabolic enzymes in the lipid pathways studied. Alcohol feeding was associated with: 1) increased hepatic free fatty acid levels, and decreased fatty acyl-CoA levels. Our gene expression data led us to conclude that these changes were associated with decreased mitochondrial fatty acid oxidation, and decreased fatty acyl-CoA synthesis, respectively; 2) Increased hepatic ceramide levels, associated with higher levels of the precursor molecules sphingosine and sphinganine; 3) Increased hepatic levels of the endocannabinoid, anandamide, which was associated with decreased expression of its catabolic enzyme, fatty acid amide hydrolase. The unique combination of lipidomic and gene expression analysis employed, allows for a better mechanistic understanding of dysregulated lipid metabolism in the development of alcoholic fatty liver disease.

Keywords

Liquid chromatography tandem mass spectrometry (LC/MS/MS), fatty acid, fatty acyl-CoA, sphingolipid, endocannabinoid, and Lieber-DeCarli diet
Introduction

Alcoholic fatty liver is generally the first presentation of alcoholic liver disease (ALD), and is thought to occur in ~20% of alcoholics (1). Studies of hepatic lipid metabolism can provide mechanistic insights into the development of alcoholic fatty liver, as well as help in identifying potential biomarkers for progression to more severe disease. Lipid metabolism has been extensively studied in the livers of alcoholic humans, and in animal models of ALD, implicating alcohol-associated alterations in several pathways, including fatty acid (FA) uptake, oxidation, and export (2,3). However until recently, technical limitations have prevented a survey of multiple lipid species in the same tissue, typically limiting studies to analyzing one lipid class, or only yielding total levels of a specific lipid class. The development of high sensitivity liquid chromatography tandem mass spectrometry (LC/MS/MS) allows for study of the so-called lipidome (i.e. the entire lipid content of tissues) in a specific model or disease of interest. Multiple lipid species can thus be assayed quantitatively in the same biological sample (4).

Initial attempts to study the lipidome of alcohol-exposed livers have recently been reported (5-7). These studies have established the feasibility of using a lipidomic approach to characterize dysregulated lipid metabolism in response to alcohol. However, their impact was limited because only changes in lipid levels were reported. Indeed, Fernando et al. concluded that an evaluation of gene expression was required to provide a mechanistic understanding of their lipid data (5). In the present study, we have taken the next step by combining lipidomic analyses of alcohol-fed mice with a survey of gene expression, targeting key enzymes in the metabolic pathways for the lipid classes studied.

Our primary objective was to undertake a targeted lipidomic analysis of plasma and liver from control and alcohol-fed mice, and to correlate observed changes in lipid levels with gene expression. Using specific internal standards, we were able to quantify levels of 62 defined lipid species by LC/MS/MS. Our analysis focused on three pathways: 1) FA metabolism (measuring free fatty acids (FFA), fatty acyl CoAs (FA-CoA), and fatty acid ethyl esters (FAEE)), 2)
sphingolipid metabolism, and 3) the endocannabinoid system. These categories were chosen because of their importance in hepatic lipid metabolism, as well as their known associations with lipotoxicity and hepatic disease. Hepatic FFA and FA-CoA are essential precursors for many lipids in the liver. FAEEs are important in the context of ALD, as they are produced by the non-oxidative metabolism of alcohol. In addition to FA metabolites, we also focused on two major lipid signaling families, sphingolipids (specifically ceramide and its precursors) and endocannabinoids (specifically anandamide (AEA) and 2-arachidonoylgllycerol (2-AG)).

Ceramides are now recognized as important signaling molecules associated with control of cell survival and replication. Dysregulated sphingolipid and ceramide metabolism have been implicated in numerous diseases, including cancer, cardiovascular disease, and ALD (6, 8, 9). As discussed below, a link between endocannabinoid signaling and fatty liver disease has been identified, with one study also suggesting an involvement in ALD (10, 11).

We have integrated the results of our targeted lipidomic study with analyses of gene expression, providing an improved mechanistic understanding of alcohol-induced changes in hepatic lipid levels. Alcohol-feeding was associated with pronounced increases in hepatic FFA levels, and conversely with a decrease in FA-CoA. Sphingolipid and ceramide levels were also increased in liver of alcohol-fed mice, as well as the endocannabinoid, AEA. Alcohol-induced changes in hepatic lipid levels are discussed in the context of our gene expression data.
Materials and Methods

Animals and alcohol feeding. All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee of Columbia University according to criteria outlined in the “Guide for the Care and use of Laboratory Animals” prepared by the National Academy of Sciences. Male C57BL/6 mice, at 3-4 months of age, were used for all studies. Mice were maintained in an environmentally controlled facility with a 12-hour light:dark cycle. All mice were maintained on a standard rodent chow diet until the beginning of the experiment, when they were randomized onto control or alcohol-containing liquid diets. Mice were fed alcohol using the Lieber-DeCarli liquid diet formulation (Bio-Serv, Frenchtown, NJ, USA). This established alcohol feeding paradigm employs nutritionally complete liquid diets, allowing mice fed the alcohol-containing diet to receive a defined volume of alcohol, and control mice to be fed an isocaloric control diet containing malto-dextrin in lieu of alcohol (12). All mice were housed singly to allow for measurement of diet consumption and to facilitate the pair-feeding of control mice. We employed a run-in period to allow the mice receiving alcohol to acclimate to alcohol feeding. This consisted of: 1 week of control liquid diet, 1 week of 2.2% v/v alcohol, 1 week of 4.5% v/v alcohol, and 2 weeks of 6.7% v/v alcohol. Body weights were measured on a weekly basis. At the end of the experiment, mice were sacrificed following a 4-5 hour fasting period. At sacrifice, blood was drawn by intra-cardiac puncture and was decanted into a tube containing 5 µl of 0.5 M EDTA, then stored on ice. Plasma was separated from cells by centrifugation for 10 min at 12,000 rpm (Model 5145 D, Eppendorf AG, Hamburg, Germany). The plasma was then transferred into a clean tube and snap frozen in liquid N2. Liver was dissected at sacrifice, weighed, and immediately snap frozen in liquid N2. All tissues were stored at -80 °C prior to analysis.

Biochemical analyses. All biochemical analyses were performed using kits and standard protocols, as recommended by the specific kit’s manufacturer. Blood alcohol content (BAC) was measured in plasma using a NAD-Alcohol dehydrogenase reagent (Sigma-Aldrich, St
Louis, MO, USA). For analysis of BAC, blood was taken between midnight and 1 am after one week of exposure to 6.7% alcohol. Alanine aminotransferase (ALT) was measured in plasma using an ALT-SL assay (Genzyme Diagnostics, Charlottetown, PE, Canada). Triglyceride measurements were made using a liquid stable triglyceride reagent (Thermo Fisher Scientific, Middleton, VA, USA). Measurements for liver triglyceride content were taken from a solution of total lipids extracted from liver homogenates using a standard Folch extraction (13). Hepatic retinyl ester concentration was determined by reverse-phase HPLC, as previously described (14).

**Liquid chromatography tandem mass spectrometry (LC/MS/MS).** A detailed description of the LC/MS/MS methodology is provided in the supplementary material. In brief, all lipid extractions were performed within one week of tissue collection. Levels of extracted lipids were measured on a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA). The identity of each lipid species was confirmed through the use of internal standards.

**RNA extraction, cDNA synthesis, and qPCR.** RNA was extracted from liver samples using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA clean-up and DNA digestion was performed on a Qiagen (Valencia, CA, USA) RNeasy column. The concentration and quality of isolated RNA was determined using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific). One microgram of purified RNA was reverse transcribed into cDNA using a high capacity cDNA RT kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative PCR was performed using a LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) with SYBR green PCR master mix (Roche Diagnostics) under uniform reaction conditions. All primers were designed using LightCycler probe design software 2.0 (Roche Diagnostics). Where more than one transcript variant was found for a given gene, a region common to all variants was used for primer design. A complete list of genes studied and the primer sequences are provided in **Supplementary Table 1**. All qPCR data analysis was performed as described by Pfaffl (15). Two reference genes were used in these studies, 18S
and cyclophilin A. Changes in expression of target genes relative to these reference genes were in good agreement; only data normalized to cyclophilin A expression are presented. While our gene expression analysis is a strength of the study, we recognize the limitation that gene expression levels do not necessarily translate to changes in protein expression. Nevertheless we feel our conclusions are supported by our lipidomic data, and are discussed within the context of the supporting literature.

**Statistical analyses and heat maps.** The data presented below were obtained from two independent studies, which were identical in experimental design. The data from both experiments were in good agreement. Consequently, combined data from both experiments are presented here. All raw data were processed in Excel (Microsoft, Redmond, WA, USA). Statistical analyses were performed using Prism 5 (GraphPad software, La Jolla, CA, USA). Data reported in the text are means ± standard deviations. Student’s t-tests were performed to determine statistical differences between control and alcohol-fed mice. A p-value < 0.05 was considered statistically significant. The multiple comparisons performed are a limitation in this study, leading to the possibility of a type 1 error. However, as noted above, it should be emphasized that two independent experiments were performed yielding confirmatory data, reinforcing the validity of our analysis. Heat maps were generated in Excel using conditional formatting; the heat maps represent the relative lipid level in alcohol-fed mice compared to control. Increasing intensity of green indicates a relative decrease in lipid level, whereas increasing intensity of red indicates an increased level. Black represents no change. White indicates that the specific lipid was assayed, but found to be undetectable in the sample.
Results

Induction of ALD in alcohol-fed mice. Following alcohol feeding, basic parameters associated with ALD were assessed in control and alcohol-fed mice (Table 1). No alcohol was detectable in the plasma of control mice, whereas alcohol-fed mice had an average BAC of 0.1 ± 0.05%, a level which is consistent with other alcohol-feeding studies in mice (16-18). At sacrifice, there were small but statistically non-significant decreases in body weight, coupled with non-significant increases in liver weight in alcohol-fed mice; this resulted in a significant increase in the liver:body weight ratio in this group. The total hepatic triglyceride content for alcohol-fed mice was significantly greater than control mice, by an average of ~15%. Similarly, alcohol-fed mice showed a significant elevation in the circulating level of triglycerides.

Consistent with the previously described effect of alcohol on hepatic retinyl ester stores in humans, the hepatic retinyl ester concentration was lower in alcohol-fed mice, relative to control mice (19). Plasma ALT levels tended to be greater than control, albeit not statistically significant. As expected, levels of the alcohol-inducible enzyme Cyp2e1 were significantly increased in alcohol-fed mice (Supplementary Figure 1).

Liver and plasma samples obtained from control and alcohol-fed mice underwent targeted lipidomic analyses by LC/MS/MS. The relative changes in lipid levels in alcohol-fed mice versus control, are described below (the absolute values for each lipid species analyzed in plasma and liver are presented in Supplementary Tables 2 and 3, respectively).

Fatty acid metabolites and gene expression analysis. The relative changes in FFAs, FA-CoAs and FAEEs levels were studied in alcohol-fed versus control mouse (Figure 1). Total FFAs in the plasma were not significantly increased by alcohol feeding, but a greater than 60% increase in hepatic FFA levels was observed (Figure 1A). In contrast to the increase in total hepatic FFAs, alcohol-fed mice showed an approximately 50% lower level of total hepatic FA-CoA. Similarly, total FA-CoA levels in the plasma were also significantly reduced (Figure 1B).
Plasma levels of total FAEE trended up in alcohol-fed mice, but this did not reach statistical significance. However, for liver, there was a ~5-fold increase in FAEE levels (Figure 1C).

In addition to measuring total levels of these lipids, we also analyzed the concentrations of individual fatty acyl species present. For plasma samples from alcohol-fed mice, statistically significant differences in FFA concentrations were observed only for the relatively unabundant myristoleic (C14:1) and docosanoic acids (22:0). There was a trend towards a decrease in plasma arachidonic acid (C20:4) levels in alcohol-fed mice, although this did not reach statistical significance owing to large standard deviations for these measures (control: 59.56 µM ± 66.59 versus alcohol: 26.85 µM ± 49.35). The majority of hepatic FFAs measured were found at higher levels in alcohol-fed mice (Figure 1D). These increases were statistically significant for 11 of 18 FFAs analyzed. The greatest increases were seen with stearic acid (18:0) and eicosapentaenoic acid (20:5), which were elevated 3-4 fold compared to control levels. When hepatic FFA levels were grouped according to the saturation of the FFAs an interesting pattern emerged (Figure 2). In absolute terms, the level of hepatic saturated FA (SFA) was not significantly changed by alcohol feeding, thus the increase in total FFA arose from significant increases in monounsaturated FA (MUFA) and polyunsaturated FA (PUFA; Figure 2A). In relative terms, the greatest increase was observed for PUFA, whereas MUFA was unchanged and SFA was significantly decreased following alcohol feeding (Figure 2B).

With regards to the relative levels of individual FA-CoA within the plasma, there was a broad decrease in FA-CoAs, which is reflected in the significant decrease in total FA-CoA measured (Figure 1E), however statistically significant decreases for individual FA-CoA species were only observed for myristic acid (C14:0) and arachidonic acid (C20:4). In contrast to the increases in hepatic FFAs observed in alcohol-fed mice, FA-CoA levels were decreased across almost all carbon chain lengths (Figure 1E). Of the 15 FA-CoA lipid species measured, 12 were significantly decreased.
The third group of FA metabolites analyzed was FAEEs (Figure 1F). As noted above, we observed a non-significant increase in total plasma FAEE. When we analyzed individual FAEE species, we found a general trend towards increased levels, though these never reached significance. However, a large statistically significant increase in total hepatic FAEE was observed following alcohol feeding. Analysis of individual FAEE species revealed that all but one of the FAEEs measured had a greater mean hepatic concentration. This increase was significant in 4 of 10 FAEEs analyzed. Strikingly, hepatic levels of ethyl stearate (C18:0) were increased almost 20-fold in alcohol-fed mice. Indeed, large increases in 18-carbon FAEEs (C18:0, C18:1, C18:2) account for almost the entire absolute increase in hepatic total FAEEs.

Hepatic gene expression profiles were determined by qPCR for the same control and alcohol-fed mice used for lipidomic analysis. To better understand the observed changes in FA metabolites, key enzymes and transcriptional regulators involved in hepatic FA metabolism were investigated. Genes which were significantly changed are presented in Figure 3, a complete table of results for our gene expression analysis is also provided (Supplementary table 4). Our analysis included genes involved in FA uptake (Cd36, Lpl, Hl, Fatp1, Fatp2, Fatp5, Fabp1), FA oxidation (Cpt1a), FA export (Apob) FA-CoA synthesis and oxidation (Acs11, Acs15, Acox1), de novo lipogenesis (Acc1, Fasn), desaturation and elongation (Scd1, Elovl5, Elovl6), triglyceride synthesis and hydrolysis (Dgat1, Dgat2, Tgh, Atgl, Hsl), and transcriptional regulation of FA metabolism (Ppara, Pparg, Srebp1c). With regard to FA uptake, we observed a significant increase in Cd36 expression in alcohol-fed mice, and decreased expression of Fatp1, Fatp2, and Fabp1. Expression of Apob, which encodes an apolipoprotein essential for export of lipid from liver into the circulation, was unchanged by alcohol feeding. Expression of Cpt1a, an important gene for FA oxidation, was significantly decreased following alcohol feeding. Similarly, Acs11, which is associated with FA-CoA synthesis, was significantly down-regulated, but Acs15 expression was not significantly different. Acox1 catalyzes the oxidation of FA-CoA and is the rate-limiting step in peroxisomal FA oxidation (20, 21); its expression in
alcohol-fed mice was unchanged compared to control. We studied three transcriptional regulators associated with lipid metabolism in the liver (Ppara, Pparg, Srebp1c). Signaling through PPARα is typically associated with increased FA oxidation. This gene was down-regulated in our alcohol-fed mice. Conversely, PPARγ, which promotes lipogenesis, was significantly increased. Genes within the de novo lipogenesis pathway are regulated in part by Srebp1c, but we observed no change in the expression of this gene, or genes it regulates (Acc1, Fasn). Scd1 encodes an enzyme that catalyzes the conversion of saturated FA to mono-unsaturated FA. This gene was significantly down-regulated in alcohol-fed mice. The elongase, Elovl5, also exhibited decreased expression following alcohol feeding. The final group of genes analyzed were those involved in triglyceride synthesis and hydrolysis. Within this group we observed a significant increase only in Dgat2 expression. The expression of all other genes tested were unchanged following alcohol feeding.

**Sphingolipid levels and gene expression analysis.** Sphingosine, SO-1P, sphinganine, SA-1P and ceramide concentrations were measured in plasma and livers of control and alcohol-fed mice (Figure 4). Alcohol exposure was associated with a significant decrease in circulating levels of sphingosine and sphinganine, although the phosphorylated forms of these sphingolipids were unchanged (Figure 4A). In the liver, the opposite phenomenon was observed; sphingosine and sphinganine were both increased in alcohol-fed mice, whereas there was a ~50% decrease in SO-1P; SA-1P was unchanged (Figure 4B). Sphingosine and sphinganine, in combination with FAs of varying chain length, are precursors for the generation of ceramide. Differences in the fatty acyl compositions of plasma and liver ceramides are presented in Figure 4D. In plasma, the total level of ceramide was equivalent in alcohol-fed and control mice (Figure 4C). For the 12 different ceramides analyzed, significant decreases were observed in N-oleoyl ceramide (C18:1) and N-docasenoyl ceramide (C22:1), although these are low-abundance lipids in the plasma ceramide pool. There was an almost 2-fold increase in total ceramide levels in liver of alcohol-fed mice (Figure 4C). Regarding specific
ceramides, of the 12 individuals analyzed, two were significantly decreased and two increased. However, these were all relatively low abundance ceramides. The increase in total ceramide apparently reflects the combined effect of small non-significant increases in the most abundant ceramides: N-palmitoyl ceramide (C16:0), N-tetracosanoyl ceramide (C24:0), and N-tetracosenoyl ceramide (C24:1), which when totaled, result in a statistically significant increase in total ceramides.

Sphingolipid and ceramide metabolism is complex, and is only now beginning to be understood (22). Our analysis of gene expression in this pathway focused on specific genes associated with: synthesis of sphinganine (Sptlc1, Sptlc2, Kdsr), the phosphorylation and dephosphorylation of sphinganine and sphingosine (Spk1, Spk2, Spp1, Spp2), SO-1P and SA-1P catabolism (Sgpl1) and ceramide synthesis (Cers2, Smpd1). Of the eight genes analyzed, only one significant change was observed (Supplementary Table 5). Specifically, expression of Kdsr in alcohol-fed mice was reduced to approximately 60% of control animals (Figure 4E).

Endocannabinoid levels and gene expression analysis. The level of the endocannabinoids AEA and 2-AG were measured in plasma and liver of control and alcohol-fed mice (Figure 5). We observed no significant difference in plasma levels of AEA or 2-AG following alcohol exposure (Figure 5A). In liver, 2-AG levels were also unchanged, though there was a more than two-fold increase in AEA observed in alcohol-fed mice (Figure 5B).

The expression of genes representing key factors in the endocannabinoid system, which were significantly changed by alcohol feeding, are shown in Figure 5C (a complete list of gene expression data is reported in Supplementary Table 6; 23). Synthesis and breakdown of AEA was studied by examining the expression of Nape-pld and Faah, respectively. Synthesis (Dagla, Daglb) and breakdown (Magl) of 2-AG, and cannabinoid receptor expression (Cb1 and Cb2) were also assessed. We observed a significant decrease in the expression of Faah, Daglb, and Magl in alcohol-fed mice. Expression levels for the other genes in this pathway were unchanged.
Discussion

We have undertaken a targeted lipidomic analysis of plasma and liver from control and alcohol-fed mice. The study’s goal was to gain an understanding of the development of fatty liver in this model of ALD. Several recent studies have used lipidomic analyses in the context of ALD, and as discussed below, many of their reported changes are in agreement with our study (5-7). We have extended the interpretation of these studies by performing a systematic analysis of gene expression in alcohol-fed mice, targeting genes within the lipid pathways studied. This unified approach allows us to interpret changes in lipid levels and gene expression together, providing a mechanistic understanding of the development of alcoholic fatty liver in mice.

Decreased FA oxidation and decreased FA-CoA synthesis is observed in livers of alcohol-fed mice. The first lipid class focused upon consisted of key FA metabolites, specifically FFAs, FA-CoAs and FAEEs. In broad terms, our data revealed striking trends for each of these lipids. There was a significant increase in total FFA and FAEE levels in liver of alcohol-fed mice, whereas total FA-CoA concentration was decreased. These findings can be understood within the context of our gene expression data. Alcohol increased expression of the FA transporter Cd36, which we take to reflect increased FA uptake from the circulation by livers of alcohol-fed mice, similar to the recent report by Ge et al (24). Expression levels of Fatp1 and Fatp2 were decreased following alcohol exposure. FATP1 and FATP2 can transport FA across plasma membranes, and both are functionally coupled with FA-CoA synthesis. Thus, decreased expression of these genes likely accounts for decreased FA-CoA levels in livers of alcohol-fed mice (25, 26). Decreased expression of Fabp1 in the livers of alcohol-fed mice was also observed. Studies of Fabp1 knock-out mice reveal a requirement for this gene in hepatic FA oxidation (27, 28). Decreased Fabp1 expression is therefore consistent with increased FFA levels in alcohol-fed mice, arising from decreased mitochondrial FA oxidation. Decreased expression of other genes associated with mitochondrial FA oxidation was also observed. Specifically, expression of Cpt1a and Acsl1 were significantly reduced. Interestingly,
peroxisomal FA oxidation was unaffected in alcohol-fed mice, as reflected by an absence of change in the expression of Acox1, which encodes the enzyme catalyzing the rate-determining step in this pathway (20, 21). No alcohol-induced changes in genes associated with de novo lipogenesis were observed (Srebp1c, Acc1, Fasn). Taken together, our data establish that the increased level of FFA in the livers of alcohol-fed mice resulted from both increased FFA uptake from the circulation and decreased mitochondrial FA oxidation. Based on the observed decreases in genes associated with FA-CoA synthesis, we also conclude that the decreased FA-CoA levels are the result of decreased FA-CoA synthesis. Both of these differences are likely associated with decreased PPARα signaling, expression of which was decreased in alcohol-fed mice, since several genes in both pathways are controlled by this transcription factor (29).

In keeping with alcohol’s established effect on liver, our alcohol-fed mice showed enlarged livers and modest, but statistically significant, increased hepatic triglyceride content. We consider our mice to be in the early stages of developing alcoholic fatty liver disease. Consistent with the literature we would expect prolonged alcohol feeding to be associated with more pronounced steatosis (30, 31). Excessive hepatic triglyceride accumulation is associated with signaling through the transcription factor PPARγ (32). Expression of Pparg was increased in livers of alcohol-fed mice, consistent with the increased triglyceride content observed. With respect to the expression of genes directly involved in hepatic triglyceride synthesis, we only observed an increase in Dgat2. Moreover, expression levels of genes involved in triglyceride hydrolysis were unchanged (Tgh, Atgl, Hsl). Investigations involving longer periods of alcohol feeding will be needed to determine the significance of decreased FA oxidation and FA-CoA synthesis as early contributing factors to ALD.

A question arises regarding the decreased FA-CoA levels observed in alcohol-fed mice in conjunction with elevated triglyceride levels. It seems counterintuitive that increased triglyceride accumulation would occur, given that FA-CoAs are an obligatory intermediate in
triglyceride synthesis. However, liver-specific knock-out of Acsl1 decreases hepatic FA-CoA levels, without protecting the mice from diet-induced steatosis (33). This result suggested that there are metabolically distinct FA-CoA pools within hepatocytes partitioned for different pathways, including one for triglyceride synthesis and one for FA oxidation. In this respect, Acsl5 is thought to catalyze the synthesis of FA-CoA in the triglyceride synthesis pathway (34, 35). In alcohol-fed mice, Acsl1 expression was significantly reduced compared to controls, whereas Acsl5 was unchanged. Though this remains to be definitively demonstrated, our data suggest that the FA-CoA pool needed for triglyceride synthesis is preserved in alcohol-fed mice, whereas the pool associated with FA oxidation is depleted.

FAEEs are non-oxidative metabolites of alcohol and are believed to be hepatotoxic (36). Their presence in multiple tissues has led to considerable research aimed at establishing FAEEs as biomarkers for chronic alcohol abuse (37). Additionally, FAEEs have also been associated with disease development in the alcoholic liver, heart, and pancreas (36, 38, 39). The molecular identities of enzymes that synthesize and degrade FAEEs are currently not established (40, 41). It is thought that multiple hepatic microsomal carboxyesterases (CESs) are able to catalyze the synthesis of FAEE; but because of this uncertainty we did not exhaustively study expression of CESs that might be involved in FAEE synthesis (41). Our results, however, do identify ethyl oleate (C18:1) as the most abundant FAEE in the plasma and livers of alcohol-fed mice. This is consistent with work done in other animal models and humans (7, 42, 43). Accumulation of FAEE in the livers of alcohol-fed mice may contribute to the development of ALD, although methods have been developed to detect these toxic lipids, their metabolism and the basis for their cytotoxic effects remains to be elucidated.

The high sensitivity of LC/MS/MS allowed quantification of individual lipid species levels within plasma and liver, allowing analysis of FA metabolites with different carbon-chain lengths. In this regard, we observed significant increases in the poly-unsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) within livers of
alcohol-fed mice. These two n-3 PUFAs are abundant in fish oils and are proposed to have beneficial effects, particularly in the cardiovascular system (44). However, there also is an extensive literature which links consumption of PUFAs with more severe ALD in humans and rodents (45-47). Our observation of elevated endogenous levels of these two PUFAs in the livers of alcohol-fed mice implies that elevations in EPA and DHA may contribute to the development of ALD.

The combined analysis of hepatic gene expression patterns and lipid levels provide insights into hepatic FFAs and their effects on gene expression. For instance, we observed a significant decrease in Elovl5 expression in the livers of alcohol-fed mice. In vivo, ELVOL5 catalyzes elongation of specific long-chain FAs, including C18:3 to C20:3. It has previously been shown that Elovl5 knock-out mice accumulate C18:3 FFA in their livers, because they cannot elongate it to C20:3 (48). Similarly, we found a decrease in hepatic Elovl5 gene expression coupled with increased C18:3 FFA following alcohol-feeding (Figure 6). In the context of ALD, this observation is significant because Elovl5 knock-out mice also develop fatty liver, though further study will be needed to establish whether this mechanism contributes to ALD (43). Also of interest was a significant decrease in hepatic Scd1 expression in alcohol-fed mice. SCD1 introduces a double-bond into saturated fatty acyl chains ranging in length from C12:0 to C19:0, yielding a monounsaturated product (49). Given that Scd1 is a known target of PPARα, the observed decrease in Ppara expression correlates with decreased Scd1 expression (45). Another likely contributing factor to decreased Scd1 expression is the fat composition of the Lieber-DeCarli diet. The FA profile of the Lieber-DeCarli liquid diet is rich in unsaturated FAs. Alcohol-fed mice take up more FFA into their livers (24), resulting in higher absolute and relative levels of hepatic unsaturated FA (Figure 2). It is established that rat primary hepatocytes down-regulate Scd1 expression when cultured with unsaturated FA of varying chain lengths, and that rats fed high amounts of unsaturated FAs also show decreased Scd1 expression (51). Interestingly, PUFAs are the most potent inhibitors of Scd1 expression and it is
this sub-type of FFA that is most increased in the livers of alcohol-fed mice. Thus, increased levels of unsaturated FFA within the livers of alcohol-fed mice, coupled with decreased Ppara expression, drives down expression of Scd1.

**Hepatic sphingolipid levels are increased following alcohol-feeding.** Hepatic levels of sphingosine, sphinganine, and total ceramides were significantly increased in alcohol-fed mice, whereas levels of SO-1P were decreased. The observed elevations in hepatic ceramides are in agreement with recently published data (6, 9). Given that sphingosine and sphinganine are both substrates for ceramide synthesis, their increased abundance in the liver correlates well with the increased ceramide levels. Unfortunately, our gene expression analysis did not provide more insight into our lipidomic data. The only significant change observed was a decrease in Kdsr expression in livers of alcohol-fed mice. The Kdsr gene product, 3-ketodihydrosphingosine reductase, is the enzyme that synthesizes sphinganine (22). Thus, we would have expected to observe a decrease in sphinganine levels, not an increase. It is possible that elevated levels of hepatic sphinganine led to a down-regulation of Kdsr via a negative feedback loop. While our gene expression data could not definitively explain the increased hepatic levels of sphingosine and sphinganine, the concomitant decrease in plasma levels of these lipids suggests that their hepatic concentration is modulated by flux between the liver and circulation, a process that may be dysregulated by alcohol exposure.

The pathophysiologic significance of increased sphingolipid levels in the livers of alcohol-fed mice requires further study. We have confirmed that hepatic ceramide levels are increased following alcohol exposure, and are the first to report that levels of the ceramide precursors, sphingosine and sphinganine, are also elevated. Reported increases in ceramide levels from different models of non-alcoholic fatty liver have lead to suggestions that ceramides are important in fatty liver disease development, though the mechanistic link between hepatic steatosis and ceramide is unclear (52-55). Increased hepatic ceramide levels have been reported following alcohol exposure *in vivo* and in H4IIEC3 cells. These increases were
associated with increased activity of acidic sphingomyelinase (Smpd1), which uses sphingomyelin as a substrate for ceramide synthesis. There was, however, no corresponding decrease in sphingomyelin reported (6, 9, 56). In our studies, we found no change in the mRNA level of Smpd1 in alcohol-fed mice.

**Hepatic anandamide is increased in alcohol-fed mice.** We observed a significant increase in AEA levels in livers of alcohol-fed mice, whereas levels of 2-AG were unchanged. Plasma levels of both lipids were also unaffected by alcohol feeding. The increased hepatic AEA levels were associated with decreased expression of Faah, which encodes an enzyme that is responsible for AEA breakdown (23). This leads us to conclude that the increased hepatic level of AEA was the result of decreased degradation. A substantial body of work links endocannabinoid signaling and hepatic steatosis (10, 57). A previous study exploring endocannabinoid signaling in alcohol-fed mice reported an increase in hepatic 2-AG levels, but not AEA (11). We found no change in the level of 2-AG in the livers of alcohol-fed mice. Nevertheless, a report by Jeong et al. suggests that an eventual disruption of this tightly regulated 2-AG concentration may be significant in ALD (11). Differences in the diets employed, or the duration of alcohol exposure, might account for the discrepancy between studies. These authors also reported that the increase in 2-AG occurred specifically in hepatic stellate cells; our study was performed in whole-liver homogenates and may therefore have lacked the resolution to detect this change. Further research will be required to resolve this issue, and determine the relative importance of AEA and 2-AG in ALD.

**Summary.** Our data supports the concept that decreased mitochondrial FA oxidation is one of the contributing factors in alcoholic fatty liver disease. Alcohol-feeding led to elevated hepatic FFA levels, coupled with decreased expression of genes associated with FA oxidation. A central mechanism underlying this observation is decreased PPARδ signaling (58, 59). As far as we are aware, we are the first to report broad decreases in FA-CoA levels within the liver of alcohol-fed mice, and that this decline is associated with decreased expression of FA-CoA
synthesizing genes. Consistent with previous reports, we observed increased ceramide levels in alcohol-fed mice (6, 9). Our data establish that this is associated with increased levels of the precursor metabolites, sphingosine and sphinganine. Dysregulated ceramide homeostasis is proposed to be an important factor in non-alcoholic fatty liver disease. Our research also indicates an important role in ALD; this will be an important question for future research to resolve. Lastly, we demonstrated an increased concentration of the endocannabinoid, AEA, in livers of alcohol-fed mice. Our data conclusively establish that the metabolic pathways for each of the three lipid classes studied are markedly dysregulated following alcohol consumption, and that this very likely contributes to the development of ALD.
References


42. Dan, L., and M. Laposata. 1997. Ethyl palmitate and ethyl oleate are the predominant fatty acid ethyl esters in the blood after ethanol ingestion and their synthesis is differentially influenced by the extracellular concentrations of their corresponding fatty acids. *Alcohol Clin Exp Res.* **21**:286-92.


Figure legends

**Figure 1.** Relative levels of plasma and liver fatty acid metabolites in alcohol-fed mice. A) The change in plasma and hepatic total FFAs are shown for alcohol-fed mice (n=11), relative to control (n=8). In control animals, plasma total FFA levels are unchanged, whereas there is a significant increase in hepatic FFA levels for alcohol-fed mice. B) Plasma and liver total FA-CoA levels are decreased in alcohol-fed mice. C) Alcohol feeding is associated with a higher level of total FAEEs within the liver, however there was no significant change in the plasma. Data graphed as mean ± SEM. Heat maps showing the relative change in plasma (left column) and hepatic lipids (right column) of different carbon-chain lengths are provided for FFAs (D), FA-CoAs (E), and FAEEs (F). * P < 0.05 alcohol-fed versus control.

**Figure 2.** Increased unsaturated fatty acid levels in alcohol-fed mice. A) Total hepatic saturated fatty acid (FFA), mono-unsaturated fatty acid (MUFA) and poly-unsaturated fatty acid (PUFA) levels in control and alcohol-fed mice, demonstrating increased levels of MUFA and PUFA following alcohol feeding. B) Relative levels of fatty acids within the liver, expressed as a percentage of total FFA. Following alcohol feeding there is a relative decrease in SFA, and a relative increase in PUFA. The relative amount of MUFA within the fatty acid pool was unchanged. Data graphed as mean ± SEM. * P < 0.05 alcohol-fed versus control.

**Figure 3.** Alcohol-feeding is associated with altered expression of genes involved in FA metabolism. Relative mRNA expression of several genes associated with FA metabolism are shown. Data graphed as mean ± SEM. * P < 0.05 alcohol-fed versus control.

**Figure 4.** Relative levels of sphingolipids in the plasma and liver of alcohol-fed mice. A) Plasma levels of sphingosine and sphinganine were significantly lower in alcohol-fed mice (n=11), relative to control (n=8), with no change in sphingosine-1-phosphate and sphinganine-1-phosphate observed. B) Liver levels of sphingosine and sphinganine were higher in alcohol-fed
mice, whereas sphingosine-1-phosphate was decreased, and sphinganine-1-phosphate was unchanged. C) Total ceramide levels were unchanged in the plasma of alcohol-fed mice, however there was a significant increase in total ceramide within the liver. D) A heat map showing relative changes in individual ceramide molecules with different fatty acyl chain lengths is shown for plasma (left column) and liver (right column). E) Kdsr expression is decreased in liver of alcohol-fed mice. All data graphed as mean ± SEM. *P < 0.05 alcohol-fed versus control.

Figure 5. Relative plasma and hepatic levels of AEA and 2-AG in alcohol-fed mice (n=11), versus control (n=8). A) Plasma levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) relative to control are shown; there was no effect of alcohol-feeding observed. B) Liver levels of AEA were significantly increased in the alcohol-fed mice, however there was no change in 2-AG observed. C) The expression of Daglb, Faah, and Magl were significantly deceased in liver of alcohol-fed mice. Data graphed as mean ± SEM. *P < 0.05 alcohol-fed versus control.

Figure 6. Decreased Elovl5 expression is associated with increased hepatic C18:3 FFA. A) Elovl5 gene expression is decreased to ~40% of control in liver of alcohol-fed mice. B) The Elovl5 substrate C18:3 is significantly increased in liver of alcohol-fed mice. Data graphed as mean ± SEM. *P < 0.05 alcohol-fed versus control.
Table 1. Physical and biochemical data

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Alcohol (n=11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.3 ± 2.2</td>
<td>26.5 ± 2.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.15 ± 0.14</td>
<td>1.20 ± 0.12</td>
<td>n.s.</td>
</tr>
<tr>
<td>Liver:body weigh ratio</td>
<td>0.041 ± 0.003</td>
<td>0.045 ± 0.002</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>Serum triglycerides (mg/dl)</td>
<td>60.6 ± 18.8</td>
<td>106.7 ± 22.8</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Liver triglycerides (mg/g)</td>
<td>45.6 ± 6.4</td>
<td>53.0 ± 1.7</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Retinyl esters (nmol/g)</td>
<td>1,441.2 ± 657.3</td>
<td>697.8 ± 293.0</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>Plasma ALT (U/l)</td>
<td>167.1 ± 45.2</td>
<td>206.0 ± 54.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Blood alcohol content (%)</td>
<td>---</td>
<td>0.10 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Cyp2e1 expression (relative units)</td>
<td>1.00 ± 0.98</td>
<td>12.21 ± 3.58</td>
<td>p &lt; 0.005</td>
</tr>
</tbody>
</table>

All data are mean ± standard deviation; p-value determined by Student’s t-test; n.s. = no significant difference.
Figure 1. Relative levels of plasma and liver fatty acid metabolites in alcohol-fed mice.
Figure 2. Increased unsaturated fatty acid levels in alcohol-fed mice.
Figure 3. Alcohol-feeding is associated with altered expression of genes involved in FA metabolism.
Figure 4. Relative levels of sphingolipids in the plasma and liver of alcohol-fed mice.
Figure 5. Relative plasma and hepatic levels of AEA and 2-AG in alcohol-fed mice, versus control.
Figure 6. Decreased Elovl5 expression is associated with increased hepatic C18:3 FFA.