Susceptibility of L-FABP<sup>−/−</sup> mice to oxidative stress in early-stage alcoholic liver<sup>§</sup>

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Abstract Chronic ethanol consumption is a prominent cause of liver disease worldwide. Dysregulation of an important lipid uptake and trafficking gene, liver-fatty acid binding protein (L-FABP), may contribute to alterations in lipid homeostasis during early-stage alcoholic liver. We have reported the detrimental effects of ethanol on the expression of L-FABP and hypothesize this may deleteriously impact metabolic networks regulating fatty acids. Male wild-type (WT) and L-FABP<sup>−/−</sup> mice were fed a modified Lieber-DeCarli liquid diet for six weeks. To assess the response to chronic ethanol ingestion, standard biochemical indicators for alcoholic liver disease (ALD) and oxidative stress were measured. Ethanol ingestion resulted in attenuation of hepatic triglyceride accumulation and elevation of cholesterol in L-FABP<sup>−/−</sup> mice. Lipidomics analysis validated multiple alterations in hepatic lipids resulting from ethanol treatment. Increased immunohistochemical staining for the reactive aldehydes 4-hydroxynonenoal and malondialdehyde were observed in WT mice ingesting ethanol; however, L-FABP<sup>−/−</sup> mice displayed prominent protein adducts in liver sections evaluated from pair-fed and ethanol-fed mice. Likewise, alterations in glutathione, thiobarbituric acid reactive substances (TBARS), 8-isoprostanes, and protein carbonyl content all indicated L-FABP<sup>−/−</sup> mice exhibit high sustained oxidative stress in the liver. These data establish that L-FABP is an indirect antioxidant protein essential for sequestering FFA and that its impairment could contribute to in the pathogenesis of ALD.—Smathers, R. L., J. J. Galligan, C. T. Shearn, K. S. Fritz, K. Mercer, M. Ronis, D. J. Orlicky, N. O. Davidson, and D. R. Petersen. Susceptibility of L-FABP<sup>−/−</sup> mice to oxidative stress in early-stage alcoholic liver. J. Lipid Res. 2013. 54: 1335–1345.

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As chronic alcohol consumption continues to be a socioeconomic burden in the United States, a growing need to further understand hepatic disease progression and to develop therapeutic intervention for dependents is rising. Recent reports have identified alcoholic liver disease as the third leading preventable cause of death, accounting for nearly 13,000 deaths in 2006 (1, 2). It has been hypothesized that early-stage ALD, namely steatosis or fatty liver, occurs as a result of the dysregulation of fatty acids (FA) through synthesis and oxidation, storage, and/or import/export mechanisms (3). Following sustained ethanol consumption, progressive stages of ALD, including hepatitis and cirrhosis, will occur in roughly 10–15% of the US population (4).

The oxidative metabolism of ethanol is known to induce hepatic stress. Coupled with the generation of reactive oxygen species (ROS), lipid peroxidation (LPO) and inflammation often parallel early-stage pathogenesis. Consumption of ethanol also alters metabolic pathways that modulate FA homeostasis in the liver (5). Recent reports have identified alterations in FA trafficking and lipid peroxidation as critical targets for modulation in the setting

Abbreviations: 4-HNE, 4-hydroxynonenoal; ACBP, acyl-CoA binding protein; ACOX1, acyl-CoA carboxylase 1; ACSL1, acyl-CoA synthetase 1 long-chain; A-FABP, adipocyte fatty acid-binding protein; ALD, alcoholic liver disease; ALT, alanine aminotransferase; BEC, blood ethanol concentration; CD36, fatty acid translocase; CPT1α, carnitine palmitoyltransferase 1A; CV, central vein; CYP2E1, cytochrome P450 2E1; FA-CoA, fatty acyl-coenzyme A; GFP, green fluorescent protein; GPx, glutathione peroxidase; GST A4, glutathione S-transferase A4; GSTπ, glutathione S-transferase pi; H&E, hematoxylin and eosin; HNF-4α, hepatocyte nuclear factor 4 alpha; HO-1, heme oxygenase-1; LCFA, long-chain fatty acid; L-FABP, liver-fatty acid binding protein; LPO, lipid peroxidation; MDA, malondialdehyde; PGC-1α, PPARγ coactivator 1 alpha; PL, phosphatidylcholine; Plin2, perilipin 2; PPAR, peroxisome proliferator-activated receptor; PT, portal triad; ROS, reactive oxygen species; SCP-2, sterol carrier protein 2; TBARS, thiobarbituric acid reactive substances; TG, triglyceride; WT, wild-type.

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of alcoholic liver injury (6–8). Liver fatty acid-binding protein (L-FABP) is highly expressed in the cytosol of hepatocytes and exhibits high affinities for lipid ligands (capable of binding two simultaneously) in the high nanomolar to low micromolar range. L-FABP is thought to function as a lipid carrier through binding ligands at surface membranes and transporting them throughout the cell to various compartments, including the nucleus, peroxisomes, mitochondria, endoplasmic reticulum, and lipid vesicles (9). Recent findings from our laboratory have identified L-FABP as a target for adduction by 4-hydroxynonenal (4-HNE), resulting in reduced ligand binding capacity and overall protein destabilization (7).

Considering the important role for L-FABP in maintaining cellular FA uptake and trafficking, we sought to understand the role of L-FABP in the progression of ALD. Utilizing L-FABP ^/^- mice and a well-characterized ethanol feeding regimen, critical alterations in hepatic lipid and redox homeostasis were observed. These results reveal novel antioxidant functions of L-FABP and suggest potential mechanisms whereby diminution of L-FABP impairs hepatic function and contributes to the pathogenesis of early-stage ALD.

MATERIALS AND METHODS

Ethanol feeding regimen

All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus and performed in accordance with published guidelines by the National Institutes of Health. L-FABP ^/^- mice were generated as described and crossed for more than 10 generations into the C57BL/6 background (10). Ten-week-old congenic male C57BL/6 [wild-type (Jackson Laboratories, Bar Harbor, ME) and L-FABP ^/^-] mice were fed a modified 45% fat-containing Lieber-DeCarli liquid diet (11–13). In the present study, mice were euthanized in the morning immediately following ad libitum feeding the previous night in order to circumvent any metabolic changes induced by fasting. At termination, the liver was harvested for whole-tissue and histological evaluation as previously described. Protein concentrations were determined utilizing a modified Lowry assay from Bio-Rad (Hercules, CA). Throughout the time course, liquid diet consumption was measured daily and body weight was measured weekly.

Biochemical assays

Serum alanine aminotransferase (ALT) activity was measured from plasma using a kit from Sekesui Diagnostics (Exton, PA). Blood ethanol concentration (BEC) was measured via head-space from plasma using a kit from Sekisui Diagnostics (Exton, PA). L-FABP with published guidelines by the National Institutes of Health.

L-FABP was measured daily and body weight was measured weekly. The liver was harvested for whole-tissue and histological evaluation as previously described (12). Microsomal lipids were derivatized without an intervening TLC step. Lipid samples and standards were measured on a GC/MS system consisting of a GC-2010 and a GC/MS-QP 2012 mass spectrometer (Shimadzu, Kyoto, Japan). In tissue samples, FFA and FA profiles were quantified utilizing an IS method (18); peak identification against known FA methyl ester (FAME) standards was utilized to generate a relative response factor (RRF). The peroxidizability index for FFA, PL, TG, and microsomes was calculated according to Lambert et al. (19).

Immunohistochemistry

Following excision, liver samples were fixed in 10% neutral-buffered formalin. Samples were processed, paraffin-embedded, and mounted on slides by the University of Colorado Research Histology Core. Slides were stained for hematoxylin and cosin (H&E) or subjected to deparaffinization and rehydration for immunohistochemical characterization with custom antibodies against 4-HNE and MDA-modified proteins (Bethyl Laboratories, Montgomery, TX) or commercial antibodies against perilipin 2 (Plin2; Fitzgerald Industries International, Acton, MA) and CYP2E1 (Millipore, Billerica, MA). Images were captured on an Olympus BX51 microscope equipped with a four megapixel Macrofire digital camera (Optronics, Goleta, CA) using the PictureFrame Application 2.3 (Optronics). All images were cropped and assembled using Photoshop CS2 (Adobe Systems, Mountain View, CA).

Immunoblotting

Proteins from whole-liver homogenates were separated by SDS-PAGE and transferred to a Hybond-P membrane (GE Healthcare, Buckinghamshire, UK). Membranes were probed with primary antibodies described in supplementary Table I. A horseradish peroxidase-conjugated secondary (Jackson Laboratories, Bar Harbor, ME) was applied, and membranes were developed using ECL-Plus Reagent (Fisher Scientific, Waltham, MA). Chemiluminescence was visualized using a Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA).

Biotin hydrazide derivatization of protein carbonyls

Protein collected from cytosolic fractions was derivatized with biotin hydrazide and stabilized with sodium borohydride as previously described (12). Blood and plasma protein (200 µg) were acid hydrolyzed in KOH and further purified utilizing 1 ml Supeleclean ENVI-18 SPE columns. Thiobarbituric acid reactive substances (TBARS) were measured in whole-liver homogenates (200 µg) according to Hartley et al. (15). GSH and GSSG were measured from whole tissue by HPLC following derivatization with 2,4-dinitrobenzene as previously described (12). Pan-GST activity was measured in whole liver homogenates according to Rinaldi et al. (16). Glutathione peroxidase (Gpx) activity was measured utilizing a specific kit from Cayman Chemical.

Lipid panel measured by GC/El-MS

Liver homogenates were extracted with chloroform-methanol (2:1, v/v) and purified by thin-layer chromatography as previously described (17). Microsomal lipids were derivatized without an intervening TLC step. Lipid samples and standards were measured on a GC/MS system consisting of a GC-2010 and a GC/MS-QP 2012 mass spectrometer (Shimadzu, Kyoto, Japan). In tissue samples, FFA and FA profiles were quantified utilizing an IS method (18); peak identification against known FA methyl ester (FAME) standards was utilized to generate a relative response factor (RRF). The peroxidizability index for FFA, PL, TG, and microsomes was calculated according to Lambert et al. (19).

Coimmunoprecipitation of protein complexes

Peroxisome proliferator-activated receptor (PPAR)a-L-FABP immunoprecipitation studies were conducted according to published work (21, 22). Briefly, 100 µg of PPARa ab (Thermo, cat#
PAI-822A) was conjugated to agarose resin, and 2 mg of pooled liver homogenate (n = 6 animals/group) was used in the immunoprecipitation. Using SDS-PAGE and immunoblotting, L-FABP and PPARα were visualized using mouse monoclonal antibodies (Abcam, Cambridge, MA).

Statistical analysis

Statistical analysis and generation of graphs were performed using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA). Differences between WT and L-FABP−/−, and control and ethanol-fed mice were assessed using a two-way ANOVA followed by Student-Newman Keuls posthoc analysis. Data are presented as mean ± SEM. Differences were considered significant if $P < 0.05$, although in many cases differences exceeded this level of probability.

RESULTS

Biochemical characterization of early-stage ALD in WT and L-FABP−/− mice

Throughout the study, food consumption was measured daily; significant, genotype-dependent effects were observed with L-FABP−/− mice at weeks 1, 3, 4, and 5 (Table 1). In agreement with previous publications using the Lieber-DeCarli diet, ethanol-treated mice displayed reduced weight gain in comparison to their isocaloric controls, with significant differences observed at five and six weeks. Blood ethanol concentrations steadily increased weekly in ethanol-consuming mice, with maximal concentrations approaching 250 mg/dl observed by six weeks of treatment. Similarly, increases in liver to body weight ratios of the ethanol-fed mice were observed with no differences due to genotype (Table 2). Data presented in Table 2 document liver injury in ethanol-fed mice by the significant 2- to 3-fold increase in plasma ALT values after six weeks of treatment with comparable changes in both WT and L-FABP−/− mice compared with their respective isocaloric controls.

| Parameter (mg/dl) | Treatment | 0   | 1   | 2   | 3   | 4   | 5   | 6   |
|-------------------|-----------|-----|-----|-----|-----|-----|-----|-----|
| L-FABP−/−         | Pair-fed  | N/A | 18.5 ± 0.42a | 17.8 ± 0.22a | 17.3 ± 0.40a | 16.3 ± 0.20a | 15.8 ± 0.19a | 16.2 ± 0.25a |
|                   | Ethanol   | N/A | 19.2 ± 0.61a | 19.0 ± 0.35a | 18.4 ± 0.43a | 17.1 ± 0.49a | 16.6 ± 0.36a | 16.4 ± 0.28a |
| L-FABP−/−         | Pair-fed  | N/A | 19.8 ± 0.33a | 18.7 ± 0.45a | 19.1 ± 0.33a | 17.9 ± 0.43a | 18.1 ± 0.45a | 18.7 ± 1.35a |
|                   | Ethanol   | N/A | 20.7 ± 0.30a | 21.7 ± 1.60a | 20.9 ± 0.93a | 18.3 ± 0.28a | 18.2 ± 0.31a | 18.3 ± 1.27a |

Data are presented as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by a Student-Newman Keuls posthoc analysis. Letter superscripts (a,b) denote a significant difference of $P < 0.05$. § denotes statistical variance is related to genotype, whereas $\$§$ denotes variance is related to ethanol treatment ($P < 0.05$) at each week. No interactions between treatment and genotype were observed. ns, nonsignificant.

Early stages of ALD are observed following six weeks of ethanol ingestion

Paraffin-embedded liver sections from treated WT and L-FABP−/− mice were stained with H&E, revealing mild lipid accumulation in the midzonal region (zone 2) in WT mice and midzonal and centrilobular regions (zones 2 and 3) in L-FABP−/− mice following ethanol consumption (Fig. 1). Immunostaining for Plin2 further demonstrated these zones of lipid accumulation (Fig. 1). In conjunction with histological analysis, hepatic and plasma triglyceride content was measured (Table 2). WT mice displayed elevated concentrations of hepatic triglycerides compared with those of the L-FABP−/− groups for both control and ethanol-treated mice. Liver triglyceride concentrations are known to fluctuate depending on feeding status; L-FABP−/− mice were protected from 48 h fasting-induced triglyceride accumulation (10). L-FABP−/− mice exhibited no difference in serum glucose and insulin concentrations following Western diet, although hepatic triglycerides were lower compared with WT controls (23). Likewise, other reports indicate that in WT mice, chronic ethanol treatment does not alter plasma glucose and insulin, or muscle glycogen synthesis in lean mice (24). Collectively, these reports support that during the early stages of ALD, insulin, glucose, and glucagon do not contribute significantly to the ethanol phenotype displayed by L-FABP−/− mice. L-FABP−/− mice displayed approximately 2-fold elevation in total hepatic cholesterol content; free cholesterol content in the liver was significantly different between the genotypes, with L-FABP−/− mice exhibiting ~5-fold higher concentrations compared with WT mice. Total plasma cholesterol was lower in ethanol-treated WT mice. Free concentrations of plasma cholesterol revealed significant genotype-dependent changes, with L-FABP−/− mice displaying nearly 2.5-fold higher concentrations compared with WT mice. While ethanol treatment resulted in significant elevations of plasma NEFA levels, marked genotype-dependent...
PUFA are more susceptible to oxidation and degradation into reactive aldehydes. Table 3 presents the component analysis of FA concentrations in WT and L-FABP−/− groups. FFA content of C18:0, C18:1, and C18:2 in WT mice, and C18:1 and C18:2 in L-FABP−/− mice were elevated due to ethanol ingestion compared with isocaloric controls. Substantial elevations in microsomal content of C16:0, C18:0, C18:2 were observed between control and ethanol-treated WT mice, and of C18:0 in the L-FABP−/− mice. Interestingly, L-FABP−/− mice exhibited attenuation in ethanol-induced microsomal C18:2 accumulation. Two-way differences were noted in hepatic NEFA content, with L-FABP−/− mice ingesting ethanol displaying a 40% increase over that observed in the WT mice treated with ethanol.

Lipid FA profile analysis of livers from WT and L-FABP−/− mice

FA are essential components of lipids and are known to exhibit important biological functions, including acting as intracellular signaling molecules involved in regulating PPAR activity (17). In addition, polyunsaturated FAs (PUFA) are more susceptible to oxidation and degradation into reactive aldehydes. Table 3 presents the component analysis of FA concentrations in WT and L-FABP−/− groups. FFA content of C18:0, C18:1, and C18:2 in WT mice, and C18:1 and C18:2 in L-FABP−/− mice were elevated due to ethanol ingestion compared with isocaloric controls. Substantial elevations in microsomal content of C16:0, C18:0, C18:2 were observed between control and ethanol-treated WT mice, and of C18:0 in the L-FABP−/− mice. Interestingly, L-FABP−/− mice exhibited attenuation in ethanol-induced microsomal C18:2 accumulation. Two-way differences were noted in hepatic NEFA content, with L-FABP−/− mice ingesting ethanol displaying a 40% increase over that observed in the WT mice treated with ethanol.

**Table 3. Standard indices of liver damage from L-FABP+/+ and L-FABP−/− mice following the Lieber-DeCarli regimen**

| Parameter                          | Pair-Fed   | Ethanol   | Pair-Fed   | Ethanol   | Genotype | Ethanol | Interaction |
|------------------------------------|------------|-----------|------------|-----------|----------|---------|-------------|
| ALT (U/l)                          | 18.41 ± 4.162a | 42.99 ± 4.523b | 13.29 ± 2.079a | 39.28 ± 6.622b | 0.2667   | <0.0001 | 0.8721      |
| Liver/body weight                  | 0.038 ± 0.0011a | 0.045 ± 0.001b | 0.038 ± 0.001a | 0.045 ± 0.001b | 0.9999   | <0.0001 | 0.8666      |
| Hepatic triglycerides (mmol/L/mg tissue) | 0.146 ± 0.021a | 0.236 ± 0.055b | 0.089 ± 0.027a | 0.147 ± 0.012b | 0.0064   | 0.0053 | 0.5249      |
| Plasma triglycerides (mmol/L)      | 0.370 ± 0.082b | 0.641 ± 0.067a | 0.660 ± 0.290b | 0.748 ± 0.171a | 0.1641   | 0.2057 | 0.5181      |
| Free hepatic cholesterol (µM/g tissue) | 0.015 ± 0.0011a | 0.015 ± 0.002a | 0.081 ± 0.011b | 0.070 ± 0.003b | <0.0001 | 0.3067 | 0.3284      |
| Total hepatic cholesterol (µM/g tissue) | 0.030 ± 0.002a | 0.032 ± 0.004b | 0.054 ± 0.006b | 0.058 ± 0.004b | <0.0001 | 0.1693 | 0.5815      |
| Free plasma cholesterol (mM)       | 0.956 ± 0.087b | 0.869 ± 0.033a | 2.223 ± 0.202b | 2.461 ± 0.160a | <0.0001 | 0.5628 | 0.2223      |
| Total plasma cholesterol (mM)      | 4.826 ± 0.218b | 3.582 ± 0.12a | 4.361 ± 0.325b | 4.418 ± 0.297a | 0.4489   | 0.0197 | 0.0113      |
| Hepatic nonesterified fatty acids (mM/g tissue) | 0.041 ± 0.003a | 0.051 ± 0.007b | 0.065 ± 0.006a | 0.103 ± 0.015b | 0.0004   | 0.0125 | 0.1188      |
| Plasma nonesterified fatty acids (mM) | 0.452 ± 0.049a | 0.869 ± 0.049b | 0.520 ± 0.031a | 0.954 ± 0.109b | 0.3022   | <0.0001 | 0.9031      |

Data are presented as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by a Student-Newman Keuls posthoc analysis. Letter superscripts (a,b) denote significant difference of P<0.05.

Fig. 1. Chronic ethanol consumption alters lipid content in WT and L-FABP−/− mice. Ethanol-consuming animals exhibit early-stage ALD following the six-week ethanol-feeding regimen as demonstrated with H&E staining and Plin2 immunohistochemistry. WT mice accumulate triglyceride in the midzonal region, and L-FABP−/− mice display mild attenuation in zones 2 and 3 of the lobule. 400× magnification.
ANOVA of the lipid panel revealed that ethanol treatment, and not the genotype, was responsible for the significant variation observed. The peroxidizability index provides an indication of the susceptibility of macromolecules, including membrane lipids, to oxidative attack (19). The variations observed. The peroxidizability index provides an indicator of antioxidant capacity in the liver; triglycerides, phosphatidylcholine, and microsomes, in which ethanol-treated L-FABP\(^{-/-}\) mice showed a 2-fold elevation in pro-oxidant susceptibility.

### Increased oxidative stress in L-FABP\(^{-/-}\) mice

It is well established that ethanol induces cellular oxidative stress by altering the redox balance of the cell and causing lipid peroxidation; cytochrome P4502E1 is proposed to be a major source of the ROS initiating this peroxidative process (25). The immunohistochemical staining presented in Fig. 2A demonstrates ethanol-induced expression of hepatic CYP2E1 in WT and L-FABP\(^{-/-}\) mice, predominantly in zones 2 and 3. The increased quantity was confirmed through Western blotting, with 1.5- to 2-fold increases in CYP2E1 protein expression (Fig. 2B). Consistent with previous reports from our laboratory, we observed a substantial increase in 4-HNE reactive proteins in zone 1 of the WT ethanol mice (26). Remarkably, prominent panlobular staining of 4-HNE-modified proteins was observed in L-FABP\(^{-/-}\) control mice with ethanol causing a redistribution of staining to zone 1. Immunohistochemical analysis further revealed ethanol treatment markedly elevated MDA-modified proteins in WT and L-FABP\(^{-/-}\) mice, while L-FABP\(^{-/-}\) control mice exhibited substantial panlobular MDA staining. To further support MDA immunohistochemistry, TBARS were measured (Table 4). Ethanol was found to increase TBARS nearly 2-fold in the WT mice, while the L-FABP\(^{-/-}\) mice displayed significantly higher TBARS content in both control and ethanol-treated animals. As an additional indicator for lipid peroxidation, 8-isoprostanes represent the pool of non-COX-derived arachidonic acid oxidation (Table 4). Ethanol elevated hepatic 8-isoprostanes in both genetic groups compared with isocaloric controls. Significant genotype-dependent differences were observed in the plasma levels of 8-isoprostanes, with control and ethanol-treated L-FABP\(^{-/-}\) mice exhibiting 2-fold higher levels compared with the respective control WT mice.

### Reduction of antioxidant capacity in L-FABP\(^{-/-}\) mice

Diminished intracellular pools of GSH accompany the induction of oxidative stress, further exacerbating the damaging effects of ROS and lipid peroxides (27). Tissues have developed a detoxification system involving both enzymatic and nonenzymatic systems, and GSH is known to conjugate reactive electrophiles, such as 4-HNE, and other oxidants to form an oxidized disulfide (GSSG) (28). To evaluate cellular oxidative stress, GSH and GSSG were quantified in WT and L-FABP\(^{-/-}\) mice (Table 4). Reductions in GSH were observed after ethanol treatment in both WT and L-FABP\(^{-/-}\) mice. The ratios of GSH to GSSG provide an indicator of antioxidant capacity in the liver; ethanol decreases the overall antioxidant capacity in WT mice.

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**Table 3. Hepatic FA composition in L-FABP\(^{+/+}\) and L-FABP\(^{-/-}\) mice following the Lieber-DeCarli regimen**

| FREE FA | Pair-Fed | Ethanol | Pair-Fed | Ethanol | Genotype | Ethanol | Interaction |
|---------|----------|---------|----------|---------|----------|---------|-------------|
| C16:0   | 31 ± 3   | 39 ± 2  | 27 ± 5   | 34 ± 5  | 0.291    | 0.115   | 0.997       |
| C18:0   | 9 ± 1\(^a\) | 13 ± 0\(^b\) | 8 ± 1   | 10 ± 2  | 0.204    | 0.034   | 0.462       |
| C18:1   | 26 ± 2\(^a\) | 45 ± 5\(^b\) | 31 ± 7\(^a\) | 52 ± 8\(^b\) | 0.461    | 0.01    | 0.963       |
| C18:2   | 100 ± 13\(^b\) | 193 ± 19\(^b\) | 77 ± 29\(^a\) | 171 ± 26\(^b\) | 0.287    | 0.001   | 0.982       |
| Peroxidizability index | 60.65 | 66.94 | 54.39 | 64.04 |          |         |             |

**Table 4. Hepatic FA composition in L-FABP\(^{+/+}\) and L-FABP\(^{-/-}\) mice following the Lieber-DeCarli regimen**

| Triglycerides | Pair-Fed | Ethanol | Pair-Fed | Ethanol | Genotype | Ethanol | Interaction |
|---------------|----------|---------|----------|---------|----------|---------|-------------|
| C16:0         | 406 ± 160 | 592 ± 40 | 349 ± 71 | 398 ± 147 | 0.301    | 0.333   | 0.565       |
| C18:0         | 64 ± 12   | 56 ± 23  | 46 ± 11  | 68 ± 26  | 0.878    | 0.699   | 0.464       |
| C18:1         | 1015 ± 202 | 1213 ± 121 | 747 ± 174 | 1034 ± 398 | 0.385    | 0.347   | 0.86        |
| C18:2         | 3025 ± 701 | 5110 ± 609 | 1725 ± 443 | 3328 ± 1243 | 0.08     | 0.041   | 0.77        |
| C20:4         | 155 ± 58  | 154 ± 9  | 72 ± 16  | 107 ± 40 | 0.047    | 0.58    | 0.55        |
| Peroxidizability index | 78.66 | 80.79 | 47.1 | 76.64 |          |         |             |

**Table 4. Hepatic FA composition in L-FABP\(^{+/+}\) and L-FABP\(^{-/-}\) mice following the Lieber-DeCarli regimen**

| Phosphatidylcholine | Pair-Fed | Ethanol | Pair-Fed | Ethanol | Genotype | Ethanol | Interaction |
|---------------------|----------|---------|----------|---------|----------|---------|-------------|
| C16:0               | 258 ± 58  | 267 ± 11 | 213 ± 58  | 228 ± 47  | 0.343    | 0.788   | 0.947       |
| C18:0               | 321 ± 40  | 415 ± 27  | 306 ± 82  | 319 ± 88  | 0.408    | 0.43    | 0.541       |
| C18:1               | 95 ± 17   | 107 ± 6  | 98 ± 28   | 148 ± 30  | 0.365    | 0.199   | 0.423       |
| C18:2               | 409 ± 91  | 598 ± 55  | 329 ± 95  | 443 ± 84  | 0.184    | 0.094   | 0.661       |
| C20:4               | 157 ± 58  | 141 ± 17  | 92 ± 24   | 129 ± 34  | 0.358    | 0.495   | 0.593       |
| Peroxidizability index | 104.28 | 76.24 | 67.38 | 75.97 |          |         |             |

**Table 4. Hepatic FA composition in L-FABP\(^{+/+}\) and L-FABP\(^{-/-}\) mice following the Lieber-DeCarli regimen**

| Microsomes | Pair-Fed | Ethanol | Pair-Fed | Ethanol | Genotype | Ethanol | Interaction |
|------------|----------|---------|----------|---------|----------|---------|-------------|
| C16:0      | 61 ± 19\(^a\) | 193 ± 17\(^b\) | 117 ± 32  | 169 ± 66  | 0.692    | 0.037   | 0.322       |
| C18:0      | 72 ± 24\(^a\) | 262 ± 16\(^b\) | 113 ± 33\(^a\) | 262 ± 63\(^b\) | 0.604    | 0.001   | 0.612       |
| C18:1      | 44 ± 13\(^a\) | 157 ± 16\(^b\) | 84 ± 25   | 134 ± 26  | 0.696    | 0.002   | 0.168       |
| C18:2      | 193 ± 64\(^b\) | 851 ± 89\(^a\) | 307 ± 78\(^b\) | 201 ± 88\(^b\) | 0.005    | 0.006   | 0.401       |
| C20:4      | 71 ± 23   | 196 ± 13  | 138 ± 39  | 822 ± 414 | 0.122    | 0.077   | 0.205       |
| Peroxidizability index | 108.41 | 98.78 | 113.45 | 219.91 |          |         |             |

Data are presented as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by a Student-Newman Keuls posthoc analysis. Letter superscripts \(^{a,b}\) denote a significant difference of \(P < 0.05\).
Fig. 2. Evaluation of the oxidative stress response in WT and L-FABP−/− mice following the Lieber-DeCarli diet regimen. Immunohistochemistry reveals ethanol- and genotype-induced elevations in CYP2E1 and the lipid peroxidation markers 4-HNE and MDA (A), 400× magnification. Immunoblot analysis (B) and relative quantification (C) of CYP2E1, HO-1, GST A4/Pi, and GPx. Different letters (a, b) indicate statistical significance of P < 0.05 or greater.

and L-FABP−/− mice, although the L-FABP−/− mice displayed even lower antioxidant capacity following ethanol challenge. As mentioned, GSH is consumed through enzymatic mechanisms to detoxify ROS in the cell (28). GSTs represent one of the major classes of proteins known to conjugate oxidized proteins and reactive aldehydes to glutathione, providing a mechanism for GSH depletion in situations of oxidative stress. Pan-GST activity was measured
in all treatment groups (Table 4). Supporting previous reports (12), WT ethanol mice were found to have elevated GST activity compared with isocaloric controls; L-FABP−/− mice displayed significant elevations in GST activity. GPxs catalyze the reduction of cellular hydroperoxides (including hydrogen peroxide), further reducing cellular GSH pools (29). In both genotypes, ethanol depleted cellular GPx activity; however, this was more substantial in L-FABP−/− mice. These data may explain the reduction in overall antioxidant capacity and elevation of LPO in L-FABP−/− mice.

Other major proteins involved in the oxidative stress response were evaluated by immunoblotting (Fig. 2B, C). HO-1 has been considered as a hallmark for the oxidative stress response, and in our model, we observed a significant ethanol-induced increase in L-FABP−/− mice. To further explain elevated GST activity and lipid peroxidation in L-FABP−/− mice, the total expression of two isoforms of GSTs (GST A4 and GSTT1) were assessed. Ethanol slightly elevated GST A4 expression in both groups; GST A4 levels were approximately 20% lower in the L-FABP−/− control mice. GSTπ expression was elevated approximately 1.4-fold in L-FABP−/− ethanol mice. The combined elevation of GST protein expression may account for increased GST activity in L-FABP−/− mice. GPx1 expression was reduced following ethanol and correlated with GPx activity in all groups.

**Elevated protein carbonylation L-FABP−/− mice**

Protein carbonylation is a well-documented consequence of chronic oxidative stress (30). Utilizing hydrazide chemistry and biotin-labeling techniques, carbonylated proteins were assessed (Fig. 3A, B). In L-FABP−/− mice, a 2- to 2.5-fold increase in total protein carbonyl content was observed compared with WT mice. Two-way ANOVA revealed significant interaction between the groups ($P < 0.01$); ethanol accounted for 4.08% of the variation ($P < 0.001$) and 92.85% was attributed to the knockout ($P < 0.001$).

**FA uptake and trafficking genes are altered in L-FABP−/− mice**

L-FABP is proposed to be essential for regulating cellular pools of FA, and genetic ablation has been shown to affect the expression of the other major lipid transporters in the liver (31). Ethanol-dependent reductions of the three cytosolic lipid transporters, namely, L-FABP, SCP-2, and ACBP, were observed (Fig. 4A). A slight elevation in SCP-2 expression was observed in pair-fed L-FABP−/− mice, which was reduced after ethanol. GFP blots validated L-FABP knockdown as previously reported (10). These data highlight ethanol-induced effects on FA trafficking.
Fig. 4. Immunoblot and coimmunoprecipitation analysis of pair- and ethanol-fed WT and L-FABP−/− mice. Immunoblots reveal alterations in genes involved in hepatic lipid uptake and trafficking (A), metabolic regulation (B), and downstream PPARα genes (C) with relative densitometry quantification (D). PPARα and HNF-4α binding activities (E) are altered, which may be further explained by the reduced ligand activation facilitated by L-FABP as demonstrated by coimmunoprecipitation analysis of PPARα and L-FABP (F). Different letters (a, b) indicate statistical significance of P < 0.05 or greater.
proteins, providing evidence for altered lipid homeostasis in early-stage ALD.

Many of these trafficking proteins are controlled by metabolic sensing transcription factors (32). Perhaps the most critical transcription factor for FA synthesis and oxidation, PPARα demonstrated a 25% reduction in total expression in ethanol-treated L-FABP−/− mice (Fig. 4B). This trend was also observed for PPARγ, and significant reductions of the coactivator protein PGC-1α were observed in ethanol-fed L-FABP−/− mice. PGC-1α is an important metabolic protein, responsible for functionally interacting with PPARs and HNF-4α to regulate activity (33). PPARγ is predominately connected with the inflammatory system and adipose tissue. However, in rodent models of ALD, PPARγ has been linked to increased lipogenesis (34). As indicators for PPARα activity in vivo, we evaluated total expression of downstream genes of PPARα (Fig. 4C). The expression of ACOX1 (peroxisomal FA oxidation) and ACLS1 (converts free FA to fatty acyl-CoA esters) was not affected by ethanol treatment. In contrast, L-FABP−/− mice demonstrated lower levels of the FA-CoA transporter CPT-1α, which is further reduced after ethanol consumption.

Ethanol ingestion alters transcription factor activity

The functionality of metabolic transcription factors were assessed in this study, revealing reductions in PPARα binding activity following ethanol ingestion (Fig. 4E). Previous reports have demonstrated decreased binding of PPARα to DNA following a four-week Lieber-DeCarli feeding regimen (35). Two-way ANOVA revealed no interaction between the groups; ethanol was determined to account for 23.07% of the total variation observed (P < 0.01). HNF4α is metabolic transcription factor involved in FA and carbohydrate metabolism, and it is also responsible for regulating PPARα expression (36). Ethanol induced HNF4α activity, which was further exacerbated in L-FABP−/− mice. Two-way ANOVA of HNF4α activity showed significant interaction between ethanol treatment and L-FABP−/− (P < 0.05); 22% of the variation observed was due to ethanol (P < 0.01) and 30% due to L-FABP−/− (P < 0.01). Alcoholics are known to have altered mitochondrial function and impaired β-oxidation of FA (37, 38). Utilizing a method from Hirschy and Verdin (39), we assessed mitochondrial β-oxidation of C14-palmitate in control and ethanol-treated animals. There were no statistical differences observed in the amount of acid-soluble metabolites formed (β-oxidation of FA) or CO2 trapped from acetyl-CoA (Kreb’s cycle) in any group (data not shown). These data suggest that this model is still early-stage, in which alterations in FA oxidation in the mitochondria are not observed.

PPARα is a ligand activated nuclear transcription factor, and it has been found to physically interact with L-FABP (21). We evaluated this protein-protein interaction in pooled samples of WT control and ethanol-treated mice (Fig. 4F). Compared with their age-matched and isocaloric control mice, the interaction between L-FABP and PPARα was reduced by nearly 50%, suggesting this reduced ligand pool in ethanol-fed mice may account for reduced transcriptional activity of PPARα in the alcoholic liver.

DISCUSSION

Animal models of early-stage ALD are often used to elucidate mechanisms of alcohol toxicity and disease progression (40, 41). Whereas the animal model used for this current investigation does replicate early ALD, encompassing steatosis and oxidative stress (12, 13, 20), it does not encompass progression from the steatosis to steatohepatitis, which would occur using more prolonged ethanol administration paradigms (42). The present report presents novel data describing the response of L-FABP−/− mice to the sustained ingestion of ethanol for six weeks. Given the proposed role of L-FABP in hepatic lipid trafficking, it was anticipated that significant alterations in lipid homeostasis would occur in mice chronically consuming ethanol. Interestingly, the data presented here reflect a complex phenotype demonstrating 1) the additive effect of high dietary fat content with ethanol and 2) the primary role of L-FABP participating as an antioxidant protein in a liver undergoing oxidative insult.

In the present study, L-FABP−/− mice were moderately protected from ethanol-induced steatosis, similar to those subjected to high-fat diets (10, 23, 43). In addition, these mice did not exhibit enhanced hepatotoxicity to ethanol challenge, as demonstrated with ALT and body weight gain (which may be attributed to the limitations of our model system). The additive effects of dietary fat and ethanol are apparent from the overall lipidomics data, including the peroxidizability of microsomal lipids and multiple measures of lipid peroxidation, demonstrating that the isocaloric high-fat diet was not benign in these mice. NEFA content, which is a known substrate for ROS attack and formation of lipid peroxides, was elevated in both isocaloric and ethanol-treated L-FABP−/− mice. CYP2E1 induction and the combined elevation in arachidonic acid within the microsomes could further contribute to the pro-oxidant effects of ethanol. All of these parameters support mechanistic details for the observed elevation in MDA and 4-HNE protein adducts in both pair-fed and ethanol-treated mice. The matter of zonation of lipid aldehyde staining may be including NADPH oxidases by Kupffer cells (44). Considering all of these factors, L-FABP−/− mice may be more likely to progress to a state of inflammation and necroinflammatory injury if the ethanol feeding regimen was sustained for a longer period of time, as in previous studies (45).

Lipidomics provided limited information on the L-FABP−/− genotype. One notable difference between the genotypes was identified from the peroxidizability index of isolated microsomes. Arachidonic acid, although highly variable, appears to be driving the peroxidizability factor and may be linked to elevated oxidative stress in L-FABP−/− mice. Previous lipid profile analysis of L-FABP−/− mice noted no significant difference in the FA components of FFA, TG, phosphatidylcholine, or phosphatidylethanolamine (10). In recent reports, rats treated with a 5% and 70% “calories from corn oil” total enteral nutrition (TEN) regimen for 21 days demonstrated that levels of FA were directly correlated with those found in the diet (17).
In the mouse model of ALD described here, these patterns were not as apparent. Approximately 60% of the FA in the diet used for these studies consisted of monounsaturated FA. Substantial differences in PUFA (C18:2, C20:4) were observed in FFA, TG, PL, and microsomal lipids in WT ethanol mice, supporting recent reports from Clugston et al. (6). These data suggest that, in mice, FAs are oxidized, newly synthesized, and/or imported from the adipose tissue. Further support has been recently established, in which deuterium-labeling methods have identified alcohol to induce reverse transport of lipids from adipose tissue back to the liver (46). This is a critical discussion point for L-FABP−/− mice, as they exhibit reduced FA uptake (43). It is unknown what concentrations of FFA induce a "critical lipotoxicity"; however, the results presented here indicate significant, ethanol-dependent elevations in FFA concentrations in our model. These ethanol-induced changes in FA components may further support mechanistic information regarding elevated lipid peroxidation in models of ALD.

Lipids are known to activate nuclear transcription factors by facilitating the release of corepressors and recruitment of coactivator proteins, including PGC-1α. Numerous FA and their derivatives, including eicosanoids and prostaglandins, serve as natural ligands for PPARs, implicating this class of proteins as critical physiological lipid sensors and regulators of lipid metabolism (47). In this report, we present novel data concerning reduced association of L-FABP to PPARα in the liver, suggesting an important mechanism for reduced activity of PPARs in ALD. Additionally, the expression lipid trafficking proteins is dramatically reduced in our model, further supporting that uptake and trafficking of FA may be an important and very critical component of metabolic dysregulation in ALD.

Oxidative stress and its damaging effects are associated with ethanol consumption, and progression of liver injury in nonalcoholic steatohepatitis (NASH) patients has been linked to FFA peroxidizability and radical attack (48, 49). While L-FABP−/− mice were mildly resistant to hepatic steatosis, a novel and more critical role for L-FABP as an indirect cellular antioxidant was observed. L-FABP−/− mice exhibited high cellular oxidative stress, as demonstrated with elevated LPO, protein carbonyls, and reduced antioxidant capacity. The antioxidant functions of hepatic and nonhepatic FABPs in vitro have been supported recently (50–53). Utilizing X-ray crystallography, Hellberg et al. have described how adipocyte FABP can sequester 4-HNE within its interior binding pocket, preventing reaction with other macromolecules (50). In addition, L-FABP can bind PUFAs and therefore modulate the availability of these FA to intracellular oxidative pathways (52).

In summary, these data suggest that loss of the primary lipid binding protein in hepatocytes renders FA available for oxidation in systems containing high levels of ROS, including ALD. Although the model utilized herein is focused on the phenotype of steatosis and oxidative stress representative of early ALD, a more prolonged regimen of ethanol administration may reveal a greater sensitivity of L-FABP−/− mice in the instance they progress to stages of steatohepatitis characterized by greater necroinflammatory injury. As noted elsewhere (40), mice administered ethanol using the Lieber-DeCarli liquid diet rarely exhibit steatohepatitis; evaluation of this phenotype was beyond the scope of this study. The present study is the first to implicate a role of L-FABP as an indirect antioxidant protein in vivo, essential for sequestering and trafficking FA to maintain cellular homeostasis.

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