Published in final edited form as:

Alcohol Clin Exp Res. 2014 February ; 38(2): 327–335. doi:10.1111/acer.12271.

RAB GTPASES ASSOCIATE WITH ISOLATED LIPID DROPLETS (LDS) AND SHOW ALTERED CONTENT AFTER ETHANOL ADMINISTRATION: POTENTIAL ROLE IN ALCOHOL-IMPAIRED LD METABOLISM

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Abstract

Background—Alcoholic liver disease is manifested by the presence of fatty liver, primarily due to accumulation of hepatocellular lipid droplets (LDs). The presence of membrane-trafficking proteins (e.g. Rab GTPases) with LDs indicates that LDs may be involved in trafficking pathways known to be altered in ethanol damaged hepatocytes. Since these Rab GTPases are crucial regulators of protein trafficking, we examined the effect ethanol administration has on hepatic Rab protein content and association with LDs.

Methods—Male Wistar rats were pair-fed Lieber-DeCarli diets for 5 to 8 weeks. Whole liver and isolated LD fractions were analyzed. Identification of LDs and associated Rab proteins was performed in frozen liver or paraffin-embedded sections followed by immunohistochemical analysis.

Results—Lipid accumulation was characterized by larger LD vacuoles and increased total triglyceride content in ethanol-fed rats. Rabs 1, 2, 3d, 5, 7 and 18 were analyzed in post-nuclear supernatant (PNS) as well as LDs. All of the Rabs were found in the PNS, and Rabs 1, 2, 5 and 7 did not show alcohol-altered content, while Rab 3d content was reduced by over 80%, and Rab 18 also showed ethanol-induced reduction in content. Rab 3d was not found to associate with LDs, while all other Rabs were found in the LD fractions, and several showed an ethanol-related decrease (Rabs 2, 5, 7, 18). Immunohistochemical analysis revealed the enhanced content of a LD-associated protein, perilipin 2 (PLIN2) that was paralleled with an associated decrease of Rab 18 in ethanol-fed rat sections.

Conclusion—Chronic ethanol feeding was associated with increased PLIN2 and altered Rab GTPase content in enriched LD fractions. Although mechanisms driving these changes are not established, further studies on intracellular protein trafficking and LD biology after alcohol administration will likely contribute to our understanding of fatty liver disease.

Keywords

Alcohol; fatty liver; lipid droplets; perilipin 2; Rab GTPase; lipid droplet trafficking

INTRODUCTION

Alcohol abuse and alcohol-induced liver disease are major health problems both in the US and worldwide. In the US alone, chronic liver disease and cirrhosis has been reported as a leading cause of mortality with nearly half of the deaths being associated with alcohol abuse and the development of alcoholic liver disease (ALD) (Minino et al., 2011). The most prevalent pathological features of ALD are the presence of fatty liver (steatosis), alcoholic hepatitis and cirrhosis. Steatosis is often thought of as an early reversible stage of ALD that is characterized, in part, by the abnormal accumulation within the hepatocyte of cytosolic lipids packaged in dynamic organelles called lipid droplets (LD) (Lieber and DeCarli, 1994). The contribution of LDs in the development and/or risk of worsening pathology in ALD remains uncharacterized and has become a potential target for beneficial intervention through the possible reversal of liver damaging events

LDs consist of a core of neutral lipids, surrounded by a monolayer of phospholipids with attached or embedded proteins. The LD proteome contains structural proteins, lipid-synthesis enzymes, lipases and membrane-trafficking proteins (Guo et al., 2009; Krahmer et al., 2009). In recent years studies have demonstrated that proteins associated with LDs can play key regulatory roles in the fate of lipid stores within cells (Brasaemle DL, 2007). It has been shown that LD-associated proteins are involved in the regulation of LD lipolysis, formation, and movement within the cell. In the liver, healthy hepatocytes can degrade LDs by several mechanisms that include the action of LD-associated lipases that directly metabolize stored lipids (Rudolf et al., 2005; Ducharme and Bickel, 2008, Marcinkiewicz et al., 2006). In addition, the growth and maturity of LDs has been related to the composition of lipid droplet coat proteins with evidence demonstrating that LDs use cellular machinery similar to that of aqueous-cored vesicles to move to targeted cellular locations and to fuse with each other (Ducharme and Bickel, 2008). Thus, it is likely that LD movement is part of a system that delivers LDs to various membrane organelles in the cell for lipid delivery and processing (Liu et al., 2007; Zehmer et al., 2009). The mechanisms that support this vesicle-based degradation process are undefined, although candidate LD-associated proteins proposed include Rab GTPases that are known to be involved in regulating membrane traffic (Fujimoto et al., 2004; Beller et al., 2006; Liu et al., 2007).

Rab GTPases are members of the wider Ras superfamily and are best known for playing essential roles in exocytic and endocytic membrane trafficking (Barbero et al., 2002; Schwartz et al., 2008; Stenmark, 2009). It has been shown that Rab proteins are involved in distinct membrane trafficking steps in endosomal and biosynthetic pathways by participating in the regulation of compartment identity, cargo delivery, protein and lipid storage/degradation, as well as specialized trafficking functions. In particular, it has been reported that Rab GTPases are associated with hepatic lipid droplets and thus may play an important role in LD biogenesis (Turro et al. 2006). However, the contribution Rab proteins have on LD trafficking and properties following ethanol administration and the role of Rab-associated LDs in pathophysiological lipid regulation in the alcoholic liver, is not known.

The liver has a central role in the regulation of fatty acid metabolism. Hepatic lipid accumulation results from an imbalance between lipid availability (from circulating lipid uptake or de novo lipogenesis) and lipid disposal (via free fatty acid oxidation or VLDL secretion). The amount of lipid that can be exported from the liver is dependent on synthesis

as well as the availability of TGs that are stored within the hepatocyte in the LD organelle (Pessayre et al., 2002). The liver does not typically function to store excessive amounts of lipids as energy reserves for the body. Therefore, the accumulation of LDs in alcoholic steatosis is likely due to disruption in LD packaging and/or secretory and endocytic trafficking pathways. Excessive LD accumulation that occurs in hepatocytes can lead to lipotoxicity with consequences of inflammation and subsequent cell death. However, little is known as to the how ethanol exposure/metabolism alters the regulation of LD accumulation and degradative processes in the hepatocyte. Given the importance of Rab GTPases in trafficking, in this study we investigated the effect of chronic ethanol consumption on the content of several Rab proteins and their association with hepatic LDs. Overall, the effect ethanol administration has on trafficking proteins associated with LDs and related changes in hepatocellular lipid and LD stores were determined.

Materials and Methods

Materials

Ethanol was purchased from Pharmaco-AAPER (Brookfield, CT). IRDye infrared secondary antibodies (Abs) and blocking buffer were from Li-COR Biosciences (Lincoln, NE). Bodipy 493/503 was obtained from Invitrogen (Carlsbad, CA). PMSF (phenylmethylsulfonyl fluoride), protease inhibitor cocktail and mouse anti-Rab7 Ab were obtained from Sigma (St. Louis, MO). Goat anti-Rab 18 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-PLIN2 Ab (10R-A117ax) was from Fitzgerald (Acton, MA). Mouse anti-actin Ab was obtained from Millipore (Billerica, MA). Rabbit polyclonal primary Abs to Rab 1, Rab 2, Rab 3, and Rab 5 were kindly provided by Mark A. McNiven (Mayo Clinic, Rochester, MN). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Animals, diet administration and tissue collection

Male Wistar rats weighing 175–200 g purchased from Charles River Labs (Portage, Michigan) were paired (8 pairs) according to weight and fed control and ethanol-containing Lieber-DeCarli diets (Lieber 1989) for 5–8 weeks as previously described (Casey et al., 2004). At the termination of the feeding period, blood was collected from the vena cava just prior to sacrifice and the livers excised, rinsed in TE buffer (10 mM Tris-HCL, 1mM EDTA, pH 7.4) and divided for the various analyses. Portions of liver tissue were either frozen immediately in liquid nitrogen and stored at –80°C until needed, placed immediately in a cryomold containing optimal cutting temperature (OCT) medium, or prepared in 10% formalin for sectioning and mounting. The remaining liver tissue was used to obtain lipid droplets and liver PNS. All animals received humane care in accordance with the guidelines established by the American Association for the Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Animal Care and Use Committee at the VA NWIHCS Research Service.

Clinical Chemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities, and alcohol content were determined by the clinical laboratory at the VA NWIHCS.

Triglycerides

The extraction of lipids from liver and isolated LDs was carried out according to the procedure of Folch et al. (1957). Aliquots of lipid extract were saponified to quantify the triglycerides using the triglyceride diagnostic kit (Thermo DMA kit, Thermo Electron Clinical Chemistry, Louisville, CO).

Histological analysis of LD number and size

Tissues were frozen in optimum cutting temperature compound (Tissue-TeK, Torrance, CA). Cut sections (5 μ m) were fixed in 4% Paraformaldehyde in 50 mM PIPES, pH 7.0 for 30 min, washed (PBS) and stained with 1 μ g/ml BODIPY fluorophore for 20 min at room temperature. After subsequent washing, the stained sections were mounted with UltraCruz mounting media containing with the nuclear stain, DAPI (Vector Laboratories, Burlingame, CA). Sections were visualized with a Zeiss 510 Meta Confocal Laser Scanning Microscope (Car Zeiss, Thornwood, NY). Quantification of LD number and size was carried out using ImageJ software (NIH, Bethesda, MA). For quantification, 3 different fields were randomly selected from each section and data pooled from sections obtained from three different sets of control and ethanol animal pairs.

Isolation of LDs

LDs were isolated from rat liver as described (Ontko et al., 1986; Yu et al., 2000) with slight modifications. Briefly, 20% liver homogenate was prepared in 60% sucrose in TE buffer (10 mM Tris-HCL, 1mM EDTA, pH 7.4) containing 1 mM PMSF and protease inhibitor cocktail. A post-nuclear supernatant (PNS) fraction was obtained by centrifugation ($1,000 \times g$) of the homogenate for 10 min. LDs were isolated by subjecting the obtained PNS fraction to ultracentrifugation through pre-chilled discontinuous sucrose gradient solutions (40%, 25% and 10% W/V) respectively, in TE buffer in a pre-cooled SW28 rotor in Beckman-L70 ultra centrifuge at 4°C for 30 min at $30,000 \times g$ with slow acceleration and no break for deceleration. The white band (lipid droplet fraction) at the top of the gradient was collected and further purified by centrifugation ($20,800 \times g$) for 10 min. The clear buffer underlying the white band was removed and the LD fraction brought up to 200 μ l with TE buffer and frozen at -70°C for Western Blot analysis.

Western Blot Analysis

Western Blot was performed as described previously (Casey et al., 2004) (Briefly, PNS and LD samples (diluted 1:20 and 1:4 respectively), were resolved on 12% reduced gels by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were blocked for 1 hour in Odyssey blocking buffer at room temperature and probed overnight with primary antibodies (1:500 dilutions in blocking buffer) at 4°C. After washing in PBS containing 1% Tween 20, the blots were incubated with IRDye-tagged secondary Abs (1:15,000 dilution) for 45 min at room temperature. Subsequent to a final wash, the immunoreactive proteins were visualized and quantified using the Odyssey Infrared Imager (Li-COR Bioscience, Lincoln, NE) and associated software. Proteins in PNS were normalized to β -actin and to PLIN2 for LDs. We chose to use PLIN2 for the LD normalization as it is a known LD associated protein and has been reported to behave as constitutive lipid droplet protein (Bickel et al., 2009).

Immunohistochemistry

Immunohistochemical staining was performed using 5 μ m-thick paraffin-embedded liver tissue sections. Briefly, embedded liver sections were deparaffinized in xylene and rehydrated in ethanol. Following deparaffinization, slides were subjected to antigen retrieval by microwaving the sections in 10mM sodium citrate buffer (pH 6) for 20 min. The tissues were rinsed once in PBS (pH 7.4), permeabilized in 2% Triton x-100/PBS, and blocked for 1 hour in 1% BSA/PBS. Sections were incubated overnight with Abs specific for PLIN2 (1:100 dilution) and Rab 18 (1:20 dilution) followed by staining with appropriate Alexa Fluor secondary Abs. Sections were mounted with vectashield mounting medium containing DAPI. Confocal images were acquired using a Zeiss 510 META laser scanning confocal microscope with staining specificity was confirmed by the absence of fluorescence signal in tissue incubated with secondary Abs alone.

Statistical Analysis

The results are expressed as mean $\pm$ SEM. Comparison between 2 groups was analyzed using the student's *t*-test. *p* values of less than 0.05 were considered significant.

RESULTS

Effect of ethanol administration on lipid-related liver parameters

Male Wistar rats were pair-fed nutritionally balanced isocaloric control or ethanol-containing liquid diets. Table 1 summarizes the body/liver weights, serum transaminase and alcohol levels, and total hepatic TG content in the livers obtained from the treated animals. At the end of experimental period, no significant variations in body weights were observed in the ethanol group when compared to their control-fed counterparts. However, the liver weight and liver to body weight ratios were found to be significantly higher (20–22%) in the ethanol-fed animals ($p < 0.05$). Assessment of hepatic function through the measure of transaminases in the serum revealed significantly higher ALT and AST levels (79% and 15%, respectively) in ethanol-fed rats compared to controls ($p < 0.05$). Additionally, alcohol feeding for the 5–8 week period increased the content (3–4 fold) of hepatic triglycerides. A similar increase was observed in the amount of LDs that was obtained from the livers of ethanol-fed animals as compared to controls.

Morphological assessment of hepatic LDs isolated from control and ethanol-fed animals

The accumulation of lipids in hepatocytes that was packaged in LDs was analyzed in livers obtained from control and ethanol-fed rats. Liver tissue sections were stained BODIPY 493/503, labeling neutral fats. Differences in the number, size and size distribution of LDs in the livers following ethanol administration were observed (Fig. 1A). Results of the quantitative analysis revealed that livers from ethanol-fed rats contained significantly ($p < 0.05$) more LDs (Fig. 1B) which were also larger in size when compared to LDs identified in the livers from control fed rats (Fig. 1C). The size of LDs varied from $0.1 \mu\text{m}^2$ to $>10 \mu\text{m}^2$, however, a significant proportion of LDs larger than $5 \mu\text{m}^2$ were detected in the livers obtained from ethanol-treated animals. This noted accumulation of LDs in ethanol-fed livers is in agreement with the total hepatic lipid data presented in Table 1.

Characterization of isolated lipid droplets

The isolated liver LD fraction was subjected to Western Blot analysis and the presence of known LD-associated proteins (PLIN2 and PLIN3) were assessed (Fig. 2). As controls, the LD blots were also probed for other cellular organelle markers for the Golgi complex (GM130), the plasma membrane (ASGPR), endoplasmic reticulum (Sec 61 α) and cytoplasm (GAPDH). The results from this protein analysis of the purified LDs clearly showed that the LD fraction contained the LD specific markers PLIN2 and PLIN3, and that the LD fraction was not found to be contaminated with cytoplasm, plasma membrane and other organelles.

Effect of ethanol administration on the association of Rab GTPases and hepatic lipid droplets

Given the potential regulatory role of Rabs in LD metabolism, we investigated the effect of ethanol on the contents of selected Rab GTPases in total liver homogenate (Fig. 3) and in the purified lipid droplet liver fraction (Fig. 4). The PNS fractions obtained from the liver homogenate and the isolated LDs were separated by SDS-gel electrophoresis and the immunoblots probed with Abs against Rab 1, Rab 2, Rab 3d, Rab 5, Rab 7 and Rab 18. Figure 3 represents the protein content of Rab GTPases detected in the liver PNS fractions, shown as representative Western Blots (Fig. 3A) and as data compiled from 5–8 independent experiments (Fig. 3B). It was determined that ethanol treatment did not alter the

protein contents of Rab 1, Rab 2, Rab 5, or Rab 7 in PNS liver fraction from the treated animals. However, the protein contents of Rab3d and Rab 18 were found to be significantly ($p < 0.05$) decreased in liver PNS after ethanol treatment. The contents of Rab GTPases in the purified liver LD fractions obtained from the control and ethanol-fed rats are presented in Figure 4. First, it was determined that all of the Rabs measured were found to be present in the liver LD fraction except for Rab 3d, which is known for exocytic function (Fig. 4A). Furthermore, the data shows that ethanol treatment did not alter Rab 1 protein content in LDs, but significantly decreased the content of Rab 2 (35.4%), Rab 5(35.6%), Rab 7 (56.0%) and Rab 18 (62.32%) contents in the LD fraction ($p < 0.05$).

Co-localization of Rab 18 with PLIN2 on LDs

Since Rab 18 is a well characterized trafficking protein and it is believed that the Rab 18 may be functionally important in LD biology, we further investigated what effect ethanol administration would have on the interaction of Rab 18 with LDs obtained from the livers of treated animals.. For localization, liver paraffin sections were stained for Rab 18 and PLIN2 and observed by confocal microscopy. As expected, the content of PLIN2 was found to be localized at the periphery of LDs (Fig. 5). Quantitative analysis demonstrated a 4–5 fold increase in PLIN2 staining in livers of ethanol-fed animals compared to controls (Fig. 5A). The content of Rab 18 was also observed at the surface of the LDs. Intriguingly, almost all of the LDs in the livers from control-fed animals were coated with Rab 18 and the Rab 18 proteins were found to colocalize with PLIN2 (Fig. 5A). However, in the livers from ethanol-fed animals, not all the LDs were found to be coated with Rab 18, especially large LDs, and importantly, and the colocalization between these two proteins was decreased after ethanol administration (Fig. 5 B&C).

DISCUSSION

Fatty liver is the most common pathological change induced by alcohol and is also one of the earliest pathological manifestations of alcoholic liver disease. We and others have shown that ethanol administration is responsible for an increase in liver to body weight ratios, enhanced liver TG levels, and the presence of steatosis in alcohol treated animals. Also, increased ALT and AST activities detected in alcohol treated animals was indicative of hepatocellular damage in the ethanol-treated group. Interestingly, it has been proposed that circulating concentrations of AST and ALT appear to give insight into the extent of fat accumulation in the injured liver (Tiikkainen et al. 2003, Sattar et al. 2004).

A key histopathological characteristic of steatosis induced by ethanol consumption is the presence of large LDs in hepatocytes (Day and Yeanman, 1994; Kharbanda et al., 2007; Mak et al., 2008). In this study, an increase in the number, size and differences in the size distribution of LDs in livers of ethanol fed rats was observed. The larger size of LDs correlated with the enhanced levels of results of total hepatic TGs detected, as TG is the form of lipid that is stored in cytoplasmic LDs. Although the physiological significance of these large LDs has yet to be formally established, their relatively low surface to volume ratio increases the efficiency of fatty acid storage as neutral lipids and reduces TG lipolysis rates relative to that of smaller LDs. The effects of these actions would be to enhance storage and suppress mobilization of intracellular fatty acids, which may provide initial protection against the cytotoxic and /or bioactive effects of fatty acids and their metabolites (Listenberger et al., 2003; Postic and Girard, 2008).

It has been reported that in hepatocytes, LDs appear to reside in a continual state of flux between formation, fusion, metabolism and vesiculation. Formation of large LDs is assumed to result from continued synthesis and accumulation of esterified lipids from lipid-modifying enzymes associated with the surrounding monolayer (Kuerschner et al., 2008) and/or the

trafficking and subsequent fusion of smaller lipid droplets (Marcinkiewicz et al., 2006; Bostrom et al., 2007; Murphy et al., 2010). However, it has not been demonstrated that the increased synthesis of lipids per se is responsible for accumulation of LDs. The fact that the number and size of LDs as well as TG levels were maintained in alcohol-treated hepatocytes for prolonged periods of time, even when “starved” in serum-free medium, as shown in our recent study (McVicker et al., 2012), suggests that LD vesiculation, degradation (lipolysis), and secretory mechanisms are indeed compromised upon alcohol exposure.

There is increasing evidence that the profiles of proteins that coat LDs play important roles in regulating LD formation, morphology, and lipolysis. PLIN2, a well known LD protein, has been shown to protect LDs from lipolysis which leads to the accumulation of LDs in the liver (Imai et al., 2007; Straub et al., 2008). In this study, there was a marked increase in the quantity of PLIN2 detected on LDs which correlated with the increase observed in the number and the size of lipid droplets following ethanol administration. Therefore, evidence presented here and by other groups suggests that the mechanism by which ethanol promotes the formation of large LD involves, in part, enhancements in PLIN2 protein content on LDs.

Many studies document the close positioning of LDs with other cellular organelles, particularly the ER, endosomes, mitochondria and peroxisomes. These organelle associations might facilitate the exchange of lipids, either for anabolic growth of LDs or for their catabolic breakdown (Murphy et al., 2009). The trafficking between LDs and organelles is mediated by a variety of different membrane-associated proteins, some of which may be unique to LDs while others appear to be components of the secretory and endocytic membrane trafficking machinery. Rab proteins (small GTPases) recruit a variety of effector proteins to mediate vesicle motility, docking and fusion to target membranes (Jordens et al., 2005). The appearance of multiple Rab proteins in the isolated LDs fraction suggests that they are a metabolically active cellular component involved in lipid trafficking in the hepatocyte.

Several studies have identified multiple ethanol-induced impairments in vesicular based transport in the hepatocytes, such as clathrin-mediated endocytosis. Because of the important role of trafficking in LDs biology and regulatory role of the Rab GTPases in trafficking, we have tested whether chronic alcohol results in alterations of Rab protein content in the liver and in isolated LDs. In this study, we found that ethanol treatment significantly decreased the total liver content of Rab 3d and Rab 18. On LDs, the protein content of several Rab proteins (Rab 2, 5, 7 and 18) was found significantly altered by chronic ethanol treatment. All of these Rabs have previously been shown to be involved in distinct membrane trafficking steps in the endosomal system. Briefly, Rab1 & 2 has been shown to be required for transport of endoplasmic reticulum (ER) derived vesicles to the Golgi complex; Rab 3d involve in exocytosis; Rab 5 is known to be involved in lipid droplet-endosome interactions; Rab 7 regulates vesicle trafficking from the early endosome, and from the late endosome to the lysosomes; and Rab 18 mediates the transfer of fatty acids or neutral lipids between LDs and the ER (Murphy et al., 2009). We have previously shown impaired protein trafficking, impaired receptor recycling and exocytosis of hepatocyte specific receptor (the asialoglycoprotein receptor) in the setting of alcoholic fatty liver (McVicker et al., 2002). Since Rab 3d is known to play an important role in exocytosis, decreased content of this protein after alcohol administration (as we observed in this study), may contribute to our previous findings related to altered endocytosis in alcoholic conditions. Additionally, the presence of Rab 5 and 7 on LDs suggests an association between endosomes, lysosomes and LDs. Importantly, Singh et al. (2009) showed that the lysosomal pathway is involved in the LD and TG breakdown process. Our results also show decreased content of Rab 2, (required for vesicular transport in the early secretory pathway), so both early and late secretory events could be affected by alcohol administration. Although

all these Rabs facilitate interactions between organelles and LDs, the detailed mechanisms of these interactions remains unclear and highlights their critical importance as regulators of lipid delivery and processing.

Among the Rab GTPases proteins investigated in our study, Rab 18 is the most characterized Rab protein that is known to be associated with LDs. In a recent study, it was shown that Rab 18 recruitment on LDs is increased with lipolytic stimulation in adipocytes, suggesting a functional role for Rab 18 in LD biology (Ozeki et al., 2005). In the present work, it was determined that only the content of Rab 18 was decreased in both liver homogenates as well as isolated LDs following ethanol treatment.. Furthermore, immunohistochemical analysis demonstrated that larger LDs have less Rab 18 and more PLIN2 content. Additionally, Rab 18 and PLIN2 colocalization patterns were found to be inversely related following ethanol administration. Ozeki et al. (2005) reported that the elimination of PLIN2 from LDs may be an important function of Rab 18. It is well known that PLIN2 is involved in the attenuation of lipolysis and that Rab 18 plays a crucial role in controlling the association between LDs and the ER. In hepatocytes, LDs are believed to form in an ER sub-compartment enriched with lipid ester-synthesizing enzymes which is followed by detachment and docking to another ER sub-compartment where lipid esters in the LDs are utilized to generate VLDL particles for secretion (Gibbons et al., 2000; Murphy, 2001). We predicted that the decreased levels of Rab 18 in the livers of ethanol-fed animals may lead to alterations in the trafficking of LDs to the ER compartments which ultimately results in hepatocellular LD accumulation and the development of alcohol-induced steatosis.

The mechanisms involved in targeting Rab18 to the surface of LDs and the molecular determinants dictating Rab18 recruitment to specific LD populations is not yet known. Martin et al. (2005) predict that Rab proteins can be associated with 30 distinct proteins, either directly or indirectly (Zerial et al., 2001). From the findings of this study, it is also possible that the Rab 18 content in ethanol LDs may influence the contents of other Rab proteins on LDs.

In summary, chronic ethanol feeding contributes to hepatic LD accumulation and the enhanced content of PLIN2 was found to correlate with the proportion of LDs in the alcoholic liver. Also, we have noted that ethanol administration interferes with LD trafficking by altering the content of Rab GTPases that are associated with hepatic lipid droplets. Furthermore, the noted decrease in the content of Rab 18 in the cytosol and in isolated LDs likely plays a key role in the changes observed in PLIN2 content on LDs and fat accumulation in the alcoholic liver. Future work examining the mechanisms involved ethanol-mediated changes to liver LDs, especially in relation to intracellular trafficking and targeting to organelles, will likely contribute to the study of LD biology and our understanding of fatty liver disease.

Acknowledgments

Grant Support: This study was supported by NIH/NIAAA; 5RC1 AA019032 and 1RO1 AA020735-01 (Drs. Casey and McNiven, Multiple PI awards) and the Department of Veterans Affairs (Casey)

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Fig 1. Ethanol feeding augments both the number and size of LDs in rat liver

A) Liver sections from control and alcohol-fed rats were embedded in OCT, before cryosectioning and staining with BODIPY 493/503. LDs were visualized by confocal microscopy (magnification: 400X). Strong lipid accumulation, characterized by larger vacuoles were observed in liver sections obtained from alcohol-fed rats. B) Analysis of the data by ImageJ software (NIH) showed that the number of LDs per microscopic field was increased over two-fold compared to controls. C) Analysis of size (volume) and quantity of LDs showed that ethanol administration resulted in both an increase in total number, with a striking increase in the number of larger LDs. Results are obtained from 2 randomly selected fields from each section, and data pooled from sections obtained from three different sets of control and ethanol animal pairs. Values are mean $\pm$ SEM; * P <0.05.

Fig 2. Characterization of purified LDs

Western blot analysis was performed with LD samples to identify the relative enrichment of PLIN 2 (ADRP) and PLIN 3 (TIP47), both known LD-associated proteins, as well as to verify the absence of markers that corresponded to other organelles [GM130, Golgi complex; ASGPR, plasma membrane; Sec 61 α , endoplasmic reticulum; GAPDH, cytosol]. Separation of LDs from liver PNS (post nuclear supernatant) and processing of LD samples for WB analysis were described in methods. PNS samples (lane 1 & 2) and isolated LDs (lane 3 & 4) were separated by SDS-PAGE and processed for immunoblotting with the indicated antibodies. The isolated LD fractions from both control and ethanol-fed animals was shown to be highly enriched in both PLIN2 and PLIN3, and did not possess proteins for known markers of other organelles. The figure is representative of 8 independent experiments.

Fig 3. Quantification of selected Rab family proteins in control and ethanol fed rat liver
 Liver post nuclear supernatant (PNS) samples were analyzed for protein content of Rab 1, Rab 2, Rab 3d, Rab 5, Rab 7 and Rab 18 levels by WB analysis. (A) Fractions of control and ethanol PNS were separated by electrophoresis and processed for immunoblotting with the various anti-Rab antibodies. (B) Quantitative data were analyzed as intensity units using the Odyssey Infrared Imager associated software. Results were normalized to β -actin, and expressed as percentage of control levels. Values are means $\pm$ SEM for 8 pairs of animals; * $P < 0.05$.

Fig 4. Quantification of selected Rab family proteins in LD fractions of control and ethanol fed rat liver

LDs from control and ethanol fed animals were analyzed for Rab 1, Rab 2, Rab 3d, Rab 5, Rab 7 and Rab 18 levels by WB analysis. LDs were purified as outlined in the Methods section; the final volume of the enriched fraction was maintained constant between the control and alcohol-fed animals and equal volumes (reflective of equal weights of liver) were loaded on the gels. (A) Representative LD blots after were separated by electrophoresis and immunoblotting with the indicated anti-Rab antibodies. Rab 3d was not detectable in the LDs. (B) Quantitative data were analyzed as intensity units using the Odyssey Infrared Imager associated software. Results were normalized to PLIN2, and expressed as percentage of control levels. Values are means $\pm$ SEM for 8 pairs of animals; * $P < 0.05$.

Fig 5. Effect of alcohol administration on Rab 18 colocalization with PLIN2 on LDs
Representative images of control (Panel A) and ethanol (Panel B) fed rat liver immunostained for PLIN2 (red) and Rab 18 (green). The individual immunostaining patterns of ADRP and Rab18 are shown in monochromatic images. The merged images show significant overlap in the ADRP and Rab18 immunofluorescence on the LDs surface, as indicated by the yellow regions in the merged images. C) Rab 18 consistently labeled smaller sized LDs usually at the periphery of larger, unlabeled LDs, or a cluster of LDs. D) The histogram represents the percentage of intensity density for ADRP and Rab 18 and protein colocalization. Results are from 3 randomly selected fields from each section, and data pooled from sections obtained from 3 sets of control and ethanol animal pairs. * $P < 0.05$.

Table 1
Effect of ethanol administration on select parameters in rats

Male Wistar rats were pair-fed nutritionally balanced isocaloric control or ethanol Lieber DeCarli diet (6.4% ethanol by volume and 36% of total calories) for 5–8 weeks.

	Control	Ethanol
Body weight (g)	369.33±6.35	371.66±12.30
Liver weight (g)	12.70±0.32	15.53±0.89*
Relative liver weight (g / 100 g body weight)	3.44±0.07	4.16±0.13*
Triglycerides (mg trig/g wet liver)	16.46 ± 1.92	55.83 ± 9.90*
Triglycerides (mg trig/100 g body weight)	56.94 ± 8.22	205.63 ± 30.76*
Serum ALT (U/L)	51.00 ± 0.81	91.29 ± 5.51*
Serum AST (U/L)	84.42 ± 3.36	97.28 ± 6.95*
Serum alcohol (mg/dl)	15.80 ± 5.8	254.40 ± 48.78*

Values for each of the individual categories are presented as means ± SEM, *n*= 8.

* *P*<0.05.