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Limited excessive voluntary alcohol drinking leads to liver dysfunction in mice

Scott A. Wegner^{1,2,3}, Katherine A. Pollard⁴, Viktor Kharazia^{1,2,3}, David Darevsky^{1,2,3}, Luz Perez⁵, Sanjoy Roychowdhury⁴, Allison Xu⁵, Dorit Ron^{1,2,3}, Laura E. Nagy⁴, and F. Woodward Hopf^{1,2,3,*}

¹Department of Neurology, University of California at San Francisco (UCSF)

²Wheeler Center for the Study of Addiction

³Alcohol and Addiction Research Group

⁴Department of Pathobiology, Lerner Research Institute, The Cleveland Clinic

⁵Diabetes Center, UCSF

Abstract

Background—Liver damage is a serious and sometimes fatal consequence of long-term alcohol intake, which progresses from early-stage fatty liver (steatosis) to later-stage steatohepatitis with inflammation and fibrosis/necrosis. However, very little is known about earlier stages of liver disruption that may occur in problem drinkers, those who drink excessively but are not dependent on alcohol.

Methods—We examined how repeated binge-like alcohol drinking in C57BL/6 mice altered liver function, as compared with a single, binge-intake session and with repeated moderate alcohol consumption. We measured a number of markers associated with early- and later-stage liver disruption, including liver steatosis, measures of liver cytochrome P4502E1 (CYP2E1) and alcohol dehydrogenase (ADH), alcohol metabolism, expression of cytokine mRNA, accumulation of 4-hydroxynonenal (4-HNE) as an indicator of oxidative stress, and ALT/AST as a measure of hepatocyte injury.

Results—Importantly, repeated binge-like alcohol drinking increased triglyceride levels in the liver and plasma, and increased lipid droplets in the liver, indicators of steatosis. In contrast, a single binge-intake session or repeated moderate alcohol consumption did not alter triglyceride levels. In addition, alcohol exposure can increase rates of alcohol metabolism through CYP2E1 and ADH, which can potentially increase oxidative stress and liver dysfunction. Intermittent, excessive alcohol intake increased liver CYP2E1 mRNA, protein and activity, as well ADH mRNA and activity. Furthermore, repeated, binge-like drinking, but not a single binge or moderate drinking, increased alcohol metabolism. Finally, repeated, excessive intake transiently elevated

*Corresponding author: F. Woodward Hopf, Department of Neurology, UCSF, 675 Nelson Rising Lane, San Francisco, CA 94158, Tel.: +1-510-418-1966, Fax: +1-415-502-7332, Frederic.Hopf@ucsf.edu.

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mRNA for the proinflammatory cytokine interleukin IL-1B and 4-HNE levels, but did not alter markers of later-stage liver hepatocyte injury.

Conclusion—Together, we provide data suggesting that even relatively limited binge-like alcohol drinking can lead to disruptions in liver function, which might facilitate the transition to more severe forms of liver damage.

Keywords

Binge; alcohol; liver; CYP2E1; ADH; induction; steatosis; tolerance

INTRODUCTION

Addiction to alcohol is a major global problem which extracts a very high personal, social and economic toll (Harwood *et al.*, 1998; Blincoe *et al.*, 2002; Mokdad *et al.*, 2004; Dawson *et al.*, 2005; Hingson *et al.*, 2005; Rehm *et al.*, 2009; Bouchery *et al.*, 2011; Sacks *et al.*, 2013; CDC, 2014; SAMHSA, 2014). There is particular interest in excessive, binge consumption of alcohol, since it likely contributes strongly to the health impact of alcohol. For example, the ~16% of adults that binge drink account for ~75% of the total economic cost of alcohol use disorders (AUDs) (CDC, 2014), and reducing intake decreases alcohol-related health risks (Dawson *et al.*, 2005; Zakhari and Li, 2007; Rehm *et al.*, 2009). Others have also emphasized the importance of problem drinking in humans who consume excessively but are not dependent on alcohol, since such drinking patterns may set the stage for the development of more serious alcohol-related problems (Esser *et al.*, 2014; Grant *et al.*, 2015).

One major negative impact of excessive drinking is the development of alcohol liver disease (ALD). ALD is a spectrum disorder, where a majority of humans with AUDs exhibit steatosis or fatty liver, while ~20% progress to the more severe steatohepatitis characterized by inflammation and fibrosis/cirrhosis, which is often fatal (Lieber, 2004; Beier and McClain, 2010; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015; Nagy *et al.*, 2016). Preclinical paradigms in rodents using non-voluntary, passive exposure methods, such as liquid diet or gavage, have shown that brief, very high alcohol exposure can result in fatty liver and changes associated with later-stage ALDs (Zeng *et al.*, 2012; Abdelmegeed *et al.*, 2013; Grasselli *et al.*, 2014), with more pronounced changes after chronic non-voluntary exposure (Lieber, 2004; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015). These passive exposure paradigms serve as important models of intermediate and later stages of ALDs, but very little is known about the earlier stages of alcohol-related liver dysfunction that could occur in problem drinkers, who drink excessively but are not physically dependent on alcohol, or on possible liver changes after voluntary alcohol drinking.

Repeated cycles of intermittent, voluntary alcohol drinking and withdrawal are associated with binge-like drinking levels, which has been used to model excessive, problem drinking in non-dependent animals; in contrast, a continuous access paradigm can model moderate intake levels (Carnicella *et al.*, 2014; Darcq *et al.*, 2015). Intermittent access to alcohol is also associated with compulsion-like alcohol consumption (Vendruscolo *et al.*, 2012; Seif *et*

al., 2013; Warnault *et al.*, 2016) and impaired cortical function and decision making (George *et al.*, 2012), suggesting face and predictive validity for other aspects of human drinking. We report here that repeated, excessive alcohol drinking significantly increased fatty liver and other markers of altered liver function, with few changes after a single binge session or repeated moderate alcohol intake. Thus, our results identify that even short durations of excessive, problem drinking can produce liver dysfunction, underscoring the clinical importance of early intervention and reducing binge-like patterns of drinking.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (Jackson Laboratories) aged 7–8 weeks were individually housed under a reverse 12-hr light/dark cycle (lights off 10 am), with ad libitum food and water, and acclimated for at least 2-wk before experiments. All experiments were conducted in accordance with procedures approved by the University of California San Francisco Institutional Animal Care and Use Committee (IACUC), and following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Voluntary Alcohol Drinking Paradigms

Mice received two-bottle choice with access to two 50-ml conical vials, one containing alcohol (v/v from 190 proof alcohol, in water) and the other water. Intake levels were determined by bottle weight, corrected for spillage, and with bottle placement alternated to control for side preference. Alcohol exposure began at noon, 2 hours into the dark cycle. Age-matched, alcohol-naïve control mice (CTL) were harvested alongside alcohol-drinking subjects for biochemistry.

Intermittent-access two-bottle choice (IA) was performed as previously described (Darcq *et al.*, 2015). Subjects had 24-hr access to 20% alcohol and water for 3-d/wk, starting Monday, Wednesday and Friday, for a total of 7 weeks. Continuous-access two-bottle choice (CA) mice had 24-hr access to 10% alcohol and water, 7-d/wk for 3 weeks (Darcq *et al.*, 2015). Thus, both CA and IA had 21 drinking sessions. Single-session access to two-bottle choice (SS) for 20% alcohol and water was performed as described (Mulligan *et al.*, 2011; Beckley *et al.*, 2016), with a single 4-hr alcohol access period. CA and SS mice were acclimated for an additional 4 and 7 weeks, respectively, to be age-matched with IA mice (Fig. 1). Importantly, mice from different groups had similar body weight at the time of experimentation.

Biochemical studies were performed after a 4-hr alcohol intake session beginning at noon, or 24-hr after withdrawal from the end of the final alcohol drinking session (24hr-WD).

A final cohort of SS and IA mice received a slightly modified age-matching schedule, where SS mice were aged-matched to IA mice undergoing their first drinking session. This was done to demonstrate that IA-drinking mice exhibited binge-level blood alcohol concentrations (BACs) during their first intake session, and to determine how drinking structure and BACs changed following a history of IA. Consumption and BAC were

measured after 0.5, 1, 2, or 4 hours access to alcohol, with only a single time point tested per mouse.

Lieber-Decarli diet

Passive alcohol exposure (23 days) was performed at the Cleveland Clinic, as previously described (Roychowdhury *et al.*, 2009a), and as approved by the Cleveland Clinic IACUC.

Triglyceride Assay

Liver triglyceride levels were assayed as previously described (Chaudhry *et al.*, 2015). Spectrophotometric quantification was performed using GPO triglyceride reagent (Pointe Scientific) run concurrently with a triglyceride standard curve (0–800 mg/dl). Raw values for liver triglycerides in CTLs were 11.0 ± 1.0 mg-triglyceride/g-tissue.

Plasma triglyceride content was determined for a subset of mice. Trunk blood was collected into heparin-coated collection tubes, plasma was isolated by centrifugation (1000g, 10-min) and stored at -20°C . Plasma was run against a glycerol standard solution using a commercial kit (Sigma), according to manufacturer protocol.

Oil Red O

Histology for Oil Red O in frozen, unfixed liver sections was performed as described (Mehlem *et al.*, 2013). Images were acquired on a 20X objective by a blinded investigator.

Western Blot Analysis

Western blot was performed as described previously (Beckley *et al.*, 2016). Briefly, 20 μg of protein was resolved on 10% Bis-TRIS acrylamide gel (Invitrogen) and transferred to nitrocellulose membrane. Membranes were then blocked in 5% milk/TBS with 0.1% Tween 20 for 60 minutes.

The primary antibodies (rabbit anti-CYP2E1: 1:30,000; Millipore; rabbit anti-GAPDH: 1:6000; SantaCruz) were applied overnight at 4°C . The following day, membranes were incubated in donkey-anti-rabbit HRP-conjugated secondary antibody (1:2000, Southern Biotech) for 1-hr at room temp. Following secondary incubation, membranes were visualized using enhanced chemiluminescent reagent (GE). Band intensities were quantified using ImageJ software (NIH).

Activity Assays

Whole liver was homogenized in a loose Dounce homogenizer in ice cold PBS and microsomes were collected by differential centrifugation. Protein concentrations were measured and normalized. CYP2E1 activity was determined by measuring the rate of hydroxylation of p-nitrophenol in 100ug of microsomal protein, as previously described (Wu and Cederbaum, 2008). For alcohol dehydrogenase (ADH) activity, whole liver was homogenized in a loose Dounce homogenizer in the ADH buffer provided in the ADH assay kit and activity quantified using a colorimetric enzymatic assay, following the manufacturer's instructions (Sigma Aldrich, St. Louis, MO).

Real-Time PCR

Total RNA was extracted using TRIzol (Ambion) reagent according to the manufacturer's protocol. One µg of RNA was reverse transcribed using an AMV reverse transcriptase system, with random primers, according to the manufacturer's protocol (Promega).

Real-time PCR was performed using SYBR green-based detection using an ABI Prism 7900HT thermocycler and Sequence Detection System 2.3 software (Applied Biosystems). Results were then analyzed using the CT method (Livak and Schmittgen, 2001), with the mRNA of interest first normalized to actin, then to CTL subjects. Primers were designed using Primer-BLAST (NCBI) and designed to span exon-exon junctions. PCR primers used in the current study are outlined in Table 1.

4-HNE Immunohistochemistry

Immunodetection of 4-HNE adducts was conducted as previously described (Roychowdhury *et al.*, 2009b). Liver tissue was formalin-fixed and paraffin-embedded, after which it was sectioned and mounted. Immunohistochemistry was then performed using rabbit antisera against 4-HNE (Alpha diagnostic 1:100). Following primary incubation, sections were conjugated using Vectastain Elite Rabbit IgG Kit (Vector Labs) and visualized using DAB substrate chromogen. Following development, slides were then counterstained using Gill's hematoxylin (Vector labs). Images were acquired on a 20X objective by a blinded investigator.

ALT/AST measurements

Plasma Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were quantified using an enzymatic assay employing the manufacturer's instructions (Sekisui Diagnostics, Lexington, MA).

Blood Alcohol Concentration

Blood alcohol concentration (BAC) measurements were performed using described methods (Weiss *et al.*, 1993; Zapata *et al.*, 2006). BAC values were determined spectrophotometrically from a standard alcohol curve (0–20 µM; 0–92.12 mg%).

Analysis

All data are shown as mean±S.E.M. In order to combine results from multiple cohorts, data were first normalized to the average from the respective control group, and expressed as % CTL. Results were analyzed using GraphPad Prism (Version 6.07, GraphPad Software) or SPSS (Version 23.0, IBM) using Student's t-test, one- or two-way ANOVA, with Bonferroni post-hoc. All non-normal results, determined using a Bartlett's test for equal variance, were analyzed using Mann-Whitney test (for 2 conditions) or Kruskal-Wallis with Dunn's posthoc (for >2 conditions).

For systemic alcohol challenge (Fig. 5), the area under the curve (AUC) was approximated using trapezoidal Reimann sums: $AUC = \frac{1}{2} (t_2 - t_1) * (BAC_{t_2} + BAC_{t_1}) + \frac{1}{2} (t_3 - t_2) * (BAC_{t_3} + BAC_{t_2})$; while, alcohol elimination was calculated from the slope of the BAC response over time ($BAC / \text{Time in hours}$). In a minor subset of subjects ($n=4$ of 45), blood collection

was not successful at all three time points. These subjects were dropped from analysis for AUC and alcohol elimination.

RESULTS

Rodent models of repeated excessive, moderate, and single binge alcohol drinking

To understand the consequences of repeated, voluntary, binge-like alcohol intake on liver function, we compared three drinking paradigms in C57BL/6 mice (Fig. 1A, see Methods). Intermittent Access (IA) and Continuous Access (CA) mice have previously been utilized to model repeated excessive drinking in non-dependent mice, or moderate alcohol intake, respectively (Carnicella *et al.*, 2014; Darceq *et al.*, 2015). Single-Session (SS) mice had a single, 4-hr drinking session, where they are known to drink binge levels of alcohol (Mulligan *et al.*, 2011; Beckley *et al.*, 2016).

IA and SS mice demonstrated very similar, binge-like alcohol intake levels after a 4-hr access period (Fig. 1B), while CA mice drank significantly less alcohol during an equivalent 4-hr period (Fig. 1B; ANOVA $F_{(2,95)}=32.45$; $p<0.0001$). IA also drank more across the 24-hr access period compared to CA mice (Fig. 1C; Mann-Whitney; $p<0.001$). Thus, only IA mice had both excessive and repeated alcohol drinking, since SS mice exhibited excessive but not repeated intake, and CA mice exhibited repeated but not excessive alcohol intake.

Repeated, excessive alcohol drinking results in increased fatty liver, a marker of steatosis

Since long-term alcohol drinking in humans is associated with fatty liver, a marker of early-stage ALD, we first examined whether the repeated excessive intake in IA mice would increase liver triglyceride levels. Given the more stable nature of steatosis (Song *et al.*, 2013), we examined triglyceride levels 24-hr following withdrawal from the final drinking session (24hr-WD). Also, because we aggregated data from multiple cohorts of mice, we expressed triglyceride levels in each mouse relative to the average triglyceride level of the CTL mice in each cohort.

Importantly, IA mice exhibited significantly increased liver triglyceride levels relative to CTL and SS (Fig. 2A; Kruskal-Wallis=15.08 across groups; $p=0.0017$), with no differences between SS or CA mice versus CTL. In addition, there was a clear trend towards greater liver triglycerides in IA over CA mice, which was significant when ran independently as a t-test ($t_{(45)}=2.140$; $p=0.038$). These results strongly suggest that repeated, excessive alcohol drinking in non-dependent individuals can induce fatty liver, which is taken as evidence of early-stage alcohol-related liver dysfunction (Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015).

To further understand the ability of repeated excessive alcohol drinking to produce fatty liver, we next examined whether IA mice would also exhibit elevated levels of plasma triglycerides. Plasma triglyceride levels in IA mice were significantly elevated relative to CTL mice (Fig. 2B; Kruskal-Wallis=12.00; $p=0.0025$; SS were not tested in these experiments). Also, there was a clear trend towards greater liver triglycerides in IA over CA mice, which was significant when ran independently as a t-test ($t_{(24)}=2.363$; $p=0.027$). Thus, similar to the increase in liver triglycerides, IA but not SS or CA mice exhibited elevations

in plasma triglyceride levels. Together, these results strongly suggest that only repeated, voluntary excessive drinking resulted in increased fatty liver, a symptom of liver dysfunction observed in the majority of humans with AUDs.

We next examined whether there were changes in fatty liver in IA mice using another method, the widely utilized Oil Red O histological stain to observe lipid droplets in the liver (Mehlem *et al.*, 2013). As shown in Fig. 2C, IA mice showed increased incidence of the red-stained lipid droplets relative to CTL, CA and SS mice. This observation concurs with the increased liver and plasma triglyceride levels in IA mice (Fig. 2A,B), and supports the hypothesis that a limited course of repeated, binge-like alcohol consumption is sufficient to increase liver fattiness.

To further examine whether lipid metabolism is altered by alcohol drinking, we examined the impact of IA, CA and SS intake on mRNA levels of SREBP1c, a lipid-synthesis-promoting transcription factor which can be activated by alcohol (You *et al.*, 2002; Eberlé *et al.*, 2004; You and Crabb, 2004; Ferré and Foulle, 2010), and PPAR α , which promotes beta-oxidation of lipids (Desvergne and Wahli, 1999). SREBP1c mRNA levels were significantly elevated in all drinking groups, and, importantly, IA SREBP1c mRNA levels were significantly greater than in all other groups (Fig. 2D, ANOVA $F_{(3,28)}=31.5$; $p<0.0001$). A different pattern was observed for PPAR α , where the only difference was that SS levels were significantly reduced compared to all other groups (Fig. 2E, ANOVA $F_{(3,28)}=9.13$; $p=0.0002$). While the implication of lowered PPAR α in SS mice is unclear (see Discussion), our SREBP1c results agree that IA intake upregulates pro-lipogenic factors, consistent with an increase in triglycerides and fatty liver.

Repeated, excessive alcohol drinking results in potentiated CYP2E1 induction after alcohol consumption

Alcohol-related liver damage is thought to be mediated, at least in part, by an increase in cytochrome P4502E1 (CYP2E1) in the liver after alcohol exposure (see Discussion). Our results above suggest that triglyceride levels in the liver were selectively impacted by repeated, excessive alcohol drinking, and thus we examined liver CYP2E1 levels in different drinking groups. Liver CYP2E1 levels were increased in SS mice (Fig. 3A; 126.7 ± 6.7 %CTL; $t_{(18)}=3.350$; $p=0.004$), and trended towards an increase in CA mice (Fig. 3B; 114.8 ± 6.28 %CTL; Mann-Whitney; $p=0.085$). In contrast, CYP2E1 protein levels in excessive-drinking IA mice were significantly increased (156.8 ± 7.9 %CTL) after a 4-hr drinking session in the (Fig. 3C; Mann-Whitney, $p<0.0001$). However, liver CYP2E1 in IA mice was not different from CTL by 24hr-WD (Fig. 3D; 106.9 ± 11.1 %CTL; Mann-Whitney; $p=0.694$), suggesting that CYP2E1 returned to baseline levels after 24-hr withdrawal (see also Roberts *et al.*, 1995a). In addition to protein changes, we also found that IA mice exhibited increased CYP2E1 mRNA levels compared to CTL subjects (Fig. 3E; Kruskal-Wallis statistic=7.843; $p=0.020$), as well as a small but significant increase in liver CYP2E1 activity in IA relative to CTL, but not CA versus control (Fig. 3F; Kruskal-Wallis statistic=7.372; $p=0.025$). Thus, intermittent, excessive drinking significantly elevated liver CYP2E1 protein levels, with overall more pronounced effect relative to other intake models tested, in agreement with our observation of increased triglycerides in IA mice (Fig. 2).

In order to better understand the changes we observed, we compared our results with those seen from the widely used Lieber-Decarli (LDc) forced-choice liquid alcohol diet, which is associated with very high alcohol exposure and significant early- and later-stage liver disruption (Lieber, 2004; Beier and McClain, 2010; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015). The increase in liver CYP2E1 with LDc (242.0 ± 7.26 % isocaloric control) was significantly greater relative to our model of repeated excessive drinking (152.2 ± 11.8 % CTL) ($t_{(6)}=6.490$; $p=0.0006$). In addition, liver CYP2E1 activity was significantly greater with LDc versus controls (CTL: $100 \pm 7.09\%$, $n=4$; LDc: $164.5 \pm 4.12\%$, $n=6$; $t_{(8)}=8.47$, $p<0.0001$), with a greater % effect relative to IA drinkers. Thus, while both alcohol models increased liver CYP2E1 levels, voluntary excessive drinking induced a more moderate increase compared to the LDc forced-alcohol diet. We note that the comparison is not exact, since LDc animals did not drink 3 days per week as with IA, but these data are still useful for comparison because of the wide usage of the LDc model.

In addition to alcohol-related changes in CYP2E1, alcohol exposure could also increase the levels or activity of alcohol dehydrogenase (ADH), another major alcohol-metabolizing enzyme. Thus, we found that ADH mRNA levels for ADH were elevated in the liver of IA mice (Fig. 4A; Kruskal-Wallis statistic=9.956; $p=0.007$). In agreement, ADH activity was significantly elevated in IA versus CTL mice, but CA and CTL were not significantly different (Fig. 4B; ANOVA $F_{(2,27)}=3.47$; $p=0.045$). Thus, IA drinking was associated with relatively greater activation of these alcohol-metabolizing enzymes relative to the other voluntary drinking models tested.

Repeated, excessive alcohol drinking results in increased metabolism of alcohol

Elevated CYP2E1 levels have been linked to elevated liver triglycerides, consistent with the elevated liver CYP2E1 (Fig. 3A) and liver and plasma triglycerides (Fig. 2) observed here after repeated excessive alcohol drinking in IA mice. To further assess the consequences of elevated liver CYP2E1, we next examined whether alcohol metabolism was enhanced in IA mice, since elevated liver CYP2E1 has also been linked to greater alcohol metabolism (Beier and McClain, 2010; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015).

We first examined Blood Alcohol Concentrations (BACs) using systemic alcohol injection (2 g/kg, i.p.) (Linsenhardt *et al.*, 2011; Lopez *et al.*, 2012; Matson and Grahame, 2013), with blood collected from each subject at three time points (0.5, 1, and 2 hr post-injection). Alcohol was injected 24-hr after the end of the previous drinking session, the 24hr-WD time point. Interestingly, only IA mice demonstrated enhanced alcohol metabolism, with decreased BAC levels at each time point after alcohol challenge relative to the other groups, and no differences among CTL, SS and CA (Fig. 5A; 2-way RM-ANOVA group: $F_{(3,41)}=6.090$; $p=0.002$). Decreased BACs in IA mice relative to all other groups was also observed when analyzing the Area Under the Curve (AUC, see Methods) (Fig. 5B; Kruskal-Wallis=16.30; $p=0.001$). In contrast, no differences in elimination rate were observed (Fig. 5C; $F_{(3,42)}=0.898$; $p=0.450$), although elimination rates closely matched those previously reported for mice (Carson and Pruett, 1996).

We next determined whether greater alcohol metabolism after repeated, excessive drinking would be observed after voluntary alcohol drinking. After a 4-hr drinking period, IA mice showed significantly greater alcohol consumption compared to CA mice (Fig. 6A; $t_{(30)}=5.740$; $p<0.0001$), but highly similar BACs (Fig. 6B; $t_{(30)}=0.236$; $p=0.814$), suggesting that IA mice have greater alcohol metabolism relative to CA mice.

We then compared alcohol intake levels and BACs in SS and IA mice after different durations of intake (0.5, 1, 2 and 4 hr). In order to not disrupt drinking, different mice were used for each time point. Alcohol consumption increased with the duration of the drinking session in both groups (Fig. 6C; time: $F_{(3,106)}=20.35$; $p<0.0001$), with similar consumption in SS and IA mice at each time point (group: $F_{(1,106)}=0.024$; $p=0.877$), and strong alcohol preference (Fig. 6D). Importantly, in SS mice, these drinking levels were associated with binge-level ($>80\text{mg}\%$) BAC values (Fig. 6E). In contrast, despite similar alcohol consumption levels in IA and SS mice, BACs were significantly lower in IA mice after 0.5, 1, 2 or 4 hours access to alcohol (Fig. 6E; group: $F_{(1,106)}=83.18$; $p<0.0001$). Although intake pattern can also contribute to the relationship between intake level and BACs (Ford *et al.*, 2013), our combined results with passive systemic alcohol injection (Fig. 5) and voluntary intake (Fig. 6) suggest that only repeated, binge-like alcohol drinking (i.e., in IA mice) increased alcohol metabolism.

Repeated, excessive alcohol drinking is associated with few markers of persistent steatohepatitis

About 20% of humans with AUDs transition to a more severe liver disease, with inflammation and structural damage to the liver (see Introduction). Thus, we examined whether we could find evidence of liver inflammation and oxidative damage. We first examined whether there were changes in mRNA transcript levels for several inflammatory cytokines associated with liver oxidation and steatohepatitis (Arteel, 2003; Ceni *et al.*, 2014). Interleukin-1B (IL-1B) mRNA levels in the liver of IA mice were significantly elevated after 4hr-intake, which returned to CTL levels by 24hr-WD (Fig. 7A; $F_{(2,38)}=3.957$; $p=0.028$), similar to CYP2E1 protein levels (Fig. 3C,D). In contrast, no significant changes were observed in the mRNA levels of interleukin-6 (IL6) (Fig. 7B; $F_{(2,38)}=1.366$; $p=0.267$) or tumor necrosis factor alpha (TNF α) (Fig. 7C; Kruskal-Wallis statistic=0.413; $p=0.813$). These findings suggest that repeated binge-like voluntary alcohol drinking led to transient elevations in IL-1B but not other inflammatory markers (Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015).

Liver oxidative stress can also be detected by examining the levels of 4-hydroxynonenal (4-HNE), a product of lipid peroxidation caused by reactive oxygen species (Roychowdhury *et al.*, 2009b), which may be produced by alcohol metabolism. 4-HNE immunoreactivity was increased in the area surrounding the hepatic central veins under all drinking conditions when compared with controls (Fig 7D; representative image per condition). However, the level of 4-HNE immunoreactivity with the voluntary drinking models was less than observed with the LDc diet (Suppl. Fig. 1). Thus, it was technically challenging to discern whether there were groups differences in 4-HNE, and we did not attempt this.

Later-stage liver disease is also characterized by injury to hepatocytes. Alanine Aminotransferase (ALT) and Aspartate Transaminase (AST) are released from the intracellular contents of damaged hepatocytes and are widely used both clinically and preclinically to detect possible disruption of liver integrity (Botros and Sikaris, 2013). Thus, we tested whether ALT and AST levels were altered in IA mice. However, we observed no differences IA mice (at 24hr-WD) and CTL in ALT (Fig. 8A; $t_{(11)}=0.095$; $p=0.926$) or AST (Fig. 8B; $t_{(11)}=0.035$; $p=0.973$), or in the ALT/AST ratio (Fig. 8C; $t_{(11)}=0.066$; $p=0.949$). These results indicate that repeated binge-like drinking may transiently alter some aspects of liver steatosis and inflammation, but did not lead to overt hepatocyte injury.

DISCUSSION

Long-term alcohol intake can produce serious liver consequences in humans, but little is known about the impact of voluntary binge-like alcohol drinking in problem drinkers, before the development of dependence. We show that repeated binge-like alcohol intake significantly increased triglyceride levels in the liver and plasma, indicating the development of fatty liver. IA-only changes in other measures of liver steatosis, including OilRedO staining and SREBP1c mRNA, were also observed. No such changes were observed after a single binge-drinking session or following repeated moderate intake, suggesting that repeated excessive drinking was required to alter liver function. In addition, liver CYP2E1 levels were significantly elevated after repeated, excessive voluntary drinking, with smaller increases in CYP2E1 after a single binge session or after repeated moderate intake. IA drinking was also associated with greater CYP2E1 and ADH mRNA levels and activity. Also, these enzymes have previously been associated with increased alcohol metabolism, and only IA-drinking mice showed reduced BAC levels after passive alcohol exposure or voluntary intake. Evidence also suggests there were transient alcohol-related increases in mRNA for the cytokine IL-1B, without changes in IL-6 or TNF α , as well as increased evidence of oxidative stress. However, there were no alterations in ALT or AST, markers of hepatocyte disruption. Thus, our results are the first to show that even limited amounts of excessive, binge-like alcohol drinking were sufficient to begin the process of liver dysfunction.

A central finding of our studies was that repeated, voluntary binge-like intake in IA mice resulted in increased triglyceride levels in both the liver and the plasma, increased lipid droplets under Oil Red O staining, and greater mRNA for the lipid synthesis enzyme SREBP1c, all indicators of greater fatty liver. These findings are likely to be of considerable health importance, since they suggest that early-stage liver dysfunction can be observed even after more limited-duration excessive drinking, which could lead to more severe consequences after more protracted intake (Lieber, 2004; Song *et al.*, 2013). Elevated liver triglycerides are widely detected after non-voluntary exposure to high alcohol levels, including both chronic (see Introduction) and brief or even a single binge-like exposure (Zeng *et al.*, 2012; Grasselli *et al.*, 2014). In contrast, we found no changes in liver triglycerides after a single binge alcohol session, where blood alcohol concentrations were >80-mg% and thus considered binge-level (Crabbe *et al.*, 2011). This could reflect differences in peak blood alcohol, for example where voluntary-drinking mice can space their intake across the single drinking session (e.g. Fig. 7C), while passive alcohol exposure

occurs in a single bolus. Moderate alcohol drinking also did not alter triglyceride levels, although it can produce other biochemical changes (Carnicella *et al.*, 2014). Furthermore, a recent study in voluntary-drinking mice showed no evidence of steatosis; the authors note that this may reflect the use of a genetically-selected high-drinking mouse line (Matson and Grahame, 2013). Thus, the repeated, excessive model examined here may circumvent this limitation, and represent the first voluntary drinking model that displays important features of early-stage ALD.

Our evidence suggests that IA mice exhibit increased SREBP1c transcript levels, an alcohol-activated lipogenic transcription factor (You *et al.*, 2002; Eberlé *et al.*, 2004; You and Crabb, 2004; Ferré and Foulfelle, 2010), relative to both SS and CA, which may provide a potential mechanism for the steatosis observed in mice undergoing repeated binge-like alcohol drinking. Since there are more moderate increases in SREBP1c and CYP2E1 with SS and CA mice, without changes in triglycerides or alcohol metabolism, this may also suggest that there could be a threshold above which these changes begin to produce liver dysfunction. However, given the complexity of the many different mechanisms in the liver that can be activated by alcohol, and the numerous interactions possible between these pathways, it can be challenging to definitively assess the causal link between different alcohol-related changes, even with the more robust liver damage associated with the LDc diet (Lieber, 2004; Beier and McClain, 2010; Ronis *et al.*, 2010; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015). In addition, we found that SS mice showed a decrease in mRNA PPAR α , an enzyme involved in lipid breakdown (Desvergne and Wahli, 1999). Although the reason for this change is unclear, we speculate it could reflect a homeostatic mechanism where alcohol acts like insulin, and PPAR α is decreased to increase lipid levels. Future studies would be required to understand this PPAR α change in SS mice, and other alcohol-related changes which are associated with altered liver fattiness in IA mice.

Repeated binge-level alcohol drinking also significantly elevated liver CYP2E1 protein and activity levels. We also observed greater CYP2E1 mRNA levels in IA drinkers; greater CYP2E1 protein has been associated with both mRNA increases (Badger *et al.*, 1993; Ronis *et al.*, 1993; Takahashi *et al.*, 1993) and post-translational mechanisms (Ronis *et al.*, 1993; Osna and Donohue, 2013; Cederbaum *et al.*, 2015). CYP2E1 metabolizes alcohol, which can increase oxidative as well as other forms of tissue damage, and alcohol-related increases in liver CYP2E1 have been linked to many aspects of liver dysfunction, including fatty liver, alcohol metabolism, inflammation and hepatocyte disruption (Lieber, 2004; Beier and McClain, 2010; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015). However, CYP2E1 does not mediate all aspects of alcohol-related liver damage (Ronis *et al.*, 2010; Leung and Nieto, 2013), and alcohol can disrupt liver function through multiple pathways, with extensive possible interactions. Nonetheless, greater IA increases in liver CYP2E1 were consistent with elevated fatty liver and alcohol metabolism only in IA mice. We did not examine how CYP2E1 contributes to alcohol-related steatosis because of the challenge of reliable long-term oral administration of inhibitors with voluntary alcohol intake, relative to passive, forced-choice models (Ronis *et al.*, 2010). Nonetheless, identifying the CYP2E1 contribution to steatosis after repeated, binge-like intake remains an important challenge we seek to address in future studies.

Alternately, pathways other than CYP2E1 may be activated only in repeated, excessive drinkers and promote liver dysfunction.

In this regard, IA drinking is also associated with greater mRNA levels and activity of ADH, another alcohol metabolizing enzyme in addition to CYP2E1. Thus, we also determined whether IA mice would exhibit greater alcohol metabolism. In fact, intake levels during the first alcohol-drinking session showed BAC levels greater than 80-mg%, thus representing binge-level intake (Crabbe *et al.*, 2011). However, after repeated sessions of excessive drinking, the same level of alcohol consumption, or a passive alcohol exposure, was associated with significantly reduced BACs, suggesting the development of greater alcohol metabolism in IA mice. Since mice exhibited binge-like BAC levels in the first drinking session, but lower BACs for the same consumption levels by the twenty-first IA drinking session, we use the term binge-like drinking to describe IA intake (see also Linsenhardt *et al.*, 2011).

CYP2E1 metabolism of alcohol is known to increase oxidative stress and contribute to inflammation and cellular damage, and passive alcohol exposure can reliably induce liver damage associated with steatohepatitis, including inflammation, liver cell disruption and fibrosis/necrosis (Lieber, 2004; Leung and Nieto, 2013; Osna and Donohue, 2013; Song *et al.*, 2013; Cederbaum *et al.*, 2015). Here, we observed transient elevations in the cytokine IL-1B, but not other cytokine markers of inflammation and oxidative stress (IL6, TNF α). In addition, immunostaining for 4-HNE, an indicator of the oxidative stress which can be produced by alcohol metabolism (Roychowdhury *et al.*, 2009b), is elevated in IA animals. Together, these provide evidence that the changes in triglycerides, alcohol metabolism, and other markers with excessive alcohol drinking are also associated with increased oxidative stress. However, 4-HNE increases were observed with all three drinking groups relative to control, but the very moderate 4-HNE changes, relative to those seen with more stringent alcohol exposure models such as the Lieber-Decarli diet (shown in Suppl. Fig. 1) make it technically challenging and unfeasible to discern whether there are group differences in 4-HNE. Nonetheless, our results taken together suggest that IA drinking overall produces more pronounced changes in different liver measures related to liver fattiness and alcohol metabolism, relative to SS and CA mice that show increases in only some of the measures examined. Additionally, ADH activity was also selectively upregulated in IA subjects, and ADH can metabolize alcohol through a similar oxidative pathway and thus could also contribute to alcohol-related oxidative stress.

In contrast to the robust induction of fatty liver in IA-drinking mice, we found little evidence that repeated binge-like drinking led to overt steatohepatitis or fibrosis/necrosis. We also found no changes in ALT or AST, indicators of liver cell injury, as well as only moderate changes related to oxidative stress (above). It is also interesting that the majority of humans with AUDs exhibit fatty liver, while only a subset (~20%) develop the steatohepatitis and fibrosis/necrosis associated with significant mortality (Beier and McClain, 2010; Nagy *et al.*, 2016). Thus, while passive-exposure models are very important for defining mechanisms by which high alcohol levels produce clinically-relevant liver damage, this change is observed across all alcohol-exposed subjects. In contrast, the voluntary, repeated binge-like drinking paradigm used here may provide a valuable model to understand the early stages of alcohol-

related liver dysfunction, and how these might facilitate the transition to more severe liver damage in a subset of individuals.

In conclusion, a majority of humans with AUD demonstrate steatosis, which can progress to steatohepatitis and fibrosis/necrosis with a high incidence of mortality, and the ALD risk is greater with higher daily alcohol intake (Zakhari and Li, 2007; Rehm *et al.*, 2009). Our results suggest that even a limited period of repeated, excessive alcohol drinking reproduced several aspects of early liver dysfunction seen in human ALDs, including fatty liver, CYP2E1 induction, and greater alcohol metabolism. Thus, the voluntary, binge-like alcohol intake model that we describe may set the stage for better understanding how voluntary drinking can promote early stages of liver dysfunction, and perhaps how these early stages transition to more advanced disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Models of Repeated Excessive, Acute Excessive or Chronic Moderate Alcohol Drinking
(A) Rectangular bars represent daily (24-hr) periods, with 7 bars for each week. Colored bars indicate alcohol availability under SS (dark gray columns), CA (light gray columns) or IA (black columns) drinking. Tissue was harvested 24-hr after the end of drinking (24hr-WD) or after 4-hr of drinking. **(B)** Intake levels after 4-hr access to alcohol in SS ($n=39$), IA ($n=38$) and CA mice ($n=21$). **(C)** 24-hr intake in IA ($n=83$) and CA mice ($n=37$), averaged across all 21 days of intake. Drinking results were aggregated from all mice excepting Figure 6. *** $p < 0.001$.

Figure 2. Repeated, binge-like drinking increased liver triglycerides, a measure of steatosis
(A) Liver triglycerides, measured after 24hr-WD and expressed relative to control in each cohort, in IA ($n=24$), CTL ($n=36$), SS ($n=9$) and CA mice ($n=23$). **(B)** Plasma triglycerides in IA ($n=15$), CTL ($n=13$), and CA mice ($n=11$). **(C)** Representative examples of Oil Red O staining from CTL, SS, CA, and IA subjects. Images are representative of duplicate images from 4–8 mice per treatment group. Scale bar is 20 μ m. **(D,E)** mRNA levels of SREBP1c **(D)** and PPAR α **(E)** ($n=8$ /condition for each). #: $p < 0.05$ SS different from all other groups; †: $p < 0.05$ different from CTL. * $p < 0.05$; ** $p < 0.01$.

Figure 3. Repeated, binge-like intake increased liver CYP2E1 induction

(A,B) Smaller but significant increases in liver CYP2E1 after 4hr-intake in (A) SS (SS: $n=10$, CTL: $n=10$), but not in (B) CA mice (CA: $n=8$, CTL: $n=8$). (C,D) Significant increase in liver CYP2E1 protein levels (C) after 4-hr of binge-like drinking in IA mice (IA: $n=17$, CTL: $n=18$), (D) but not after 24-hr withdrawal (IA: $n=8$, CTL: $n=7$). (E) Transcript levels for CYP2E1 (CTL: $n=14$; IA after 4hr-intake: $n=12$; IA after 24hr-WD: $n=10$). (F) CYP2E1 enzymatic activity after 4-hr intake ($n=10$ /condition). * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$.

Figure 4. Liver ADH transcript levels and enzymatic activity is increased in IA subjects
(A) Transcript levels of ADH are significantly elevated in IA subjects 24-hr following alcohol exposure (CTL: $n=14$; IA after 4hr-intake: $n=12$; IA after 24hr-WD: $n=10$). **(B)** Liver ADH enzymatic activity after 4-hr intake ($n=10$ /condition). * $p < 0.05$; ** $p < 0.001$.

Figure 5. Only IA mice showed evidence for increased metabolism of alcohol

(A) BAC levels after systemic (i.p.) injection of 2 g/kg alcohol in IA ($n=17$), CTL ($n=15$), SS ($n=10$) and CA mice ($n=8$). Symbols for SS and CA are displaced slightly for clarity. Some error bars smaller than the symbols shown. (B) The area under the curve (AUC, see Methods). (C) Elimination rate, the decrease in BAC levels across 0.5 to 2 hours after alcohol injection (see Methods). * $p < 0.05$; ** $p < 0.01$.

Figure 6. IA mice show evidence of greater alcohol metabolism during voluntary intake (A,B) After a 4-hr drinking session, IA mice ($n=22$) showed significantly greater alcohol intake (A) with no differences in BACs (B) relative to CA mice ($n=11$). (C–E) IA mice showed (C) similar alcohol intake levels as SS mice after 0.5, 1, 2 and 4 hr of access to alcohol (SS: $n=11,12,12,23$; IA: $n=14,13,13,16$), with (D) high preference for alcohol across the majority of the drinking session in both groups, but (E) BACs were significantly lower in IA mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 7. IA drinking and markers of inflammation in the liver

(A) IA mice showed transient increases in liver mRNA transcripts of IL-1B after 4hr-intake ($n=14$) compared with CTL mice ($n=17$), which returned to basal levels after 24hr-WD ($n=10$). (B,C) IA drinking did not alter IL-6 (B) or TNFa (C) transcript levels. * $p < 0.05$. (D) Representative examples of 4-HNE immunoreactivity from each condition. 4-HNE signal was increased surrounding the hepatic central veins under alcohol drinking conditions (black arrow: SS, CA, IA) compared with CTL conditions (white arrow). Scale bar is 20 μ m. Images are representative of duplicate images from 8–9 mice per treatment group.

Figure 8. Repeated, binge-like alcohol drinking in IA mice did alter ALT or AST levels, measures of steatohepatitis

Hepatocyte injury was assessed by (A) ALT levels, (B) AST levels, or (C) the ALT/AST ratio in IA ($n=7$) versus CTL mice ($n=6$).

Table 1

Forward (F) and reverse (R) primers used for analysis of mRNA levels.

CYP2E1	F: 5' - CTGCAGTCCGAGACAGGATG -3' R: 5' - CAACTGTACCCTTGGGGATGA -3'
ADH	F: 5' - TGACACCATGACTTCTGCCC -3' R: 5' - TACGACGACGCTTACACCAC -3'
IL-1B	F: 5' - GCCACCTTTTGACAGTGATGAG -3' R: 5' - GACAGCCCAGGTCAAAGGTT-3'
IL6	F: 5' - TCTGCAAGAGACTTCCATCCAGT-3' R: 5' - GTGAAGTAGGGAAGGCCGTG-3'
TNF α	F: 5' - CCCTCACACTCAGATCATCTTCT-3' R: 5' - GCTACGACGTGGGCTACAG-3'
ACTIN	F: 5' - ATCAAGATCATTGCTCCTCCTGA-3' R: 5' - ACGCAGCTCAGTAACAGTCC -3'
PPAR α	F: 5' - GACGTTGTCATCACAGCTTAG-3' R: 5' - CTTTCCAGGTCATCTGCCTC-3'
SREBP1C	F: 5' - GGAAGCTGTCGGGGTAGC-3' R: 5' - GCTGGAGCATGTCTTCAAATGT-3'