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Lipin deactivation after acetaminophen overdose causes phosphatidic acid accumulation in liver and plasma in mice and humans and enhances liver regeneration

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1. INTRODUCTION

New treatments and biomarkers for acetaminophen (APAP) hepatotoxicity are needed. APAP overdose is responsible for approximately 50% of all acute liver failure (ALF) cases in the United States, making it the leading cause of ALF and ALF-related deaths (Lee, 2008). It has been reported that 50–80,000 emergency department visits, 20–30,000 hospitalizations, and approximately 500 deaths in the US can be attributed to APAP each year (Nourjah et al., 2006; Budnitz et al., 2011). Currently, the only effective treatment for APAP overdose is *N*-acetylcysteine (NAC). Although NAC is protective if administered during the early metabolism phase, the protection is significantly reduced 16 h after APAP overdose and completely lost by 24 h (Rumack et al., 1981). Unfortunately, many patients do not present until later time points. Craig et al. (2012) found that nearly half of all APAP overdose patients seen at the Royal Infirmary of Edinburgh between 1992 and 2008 presented >24 h after overdose, and late presentation was associated with poor outcome. The major treatment option at that point is a liver transplant, but current biomarkers for liver

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injury and function do not predict patient outcome or, therefore, the need for a transplant (Antoine et al., 2012; McGill et al., 2014a; McGill, 2016).

A logical approach to treatment of APAP overdose in late-presenting patients is to enhance liver regeneration. The liver has the capacity to regenerate approximately 80% of its functional mass after injury, and liver regeneration is thought to be a critical determinant of outcome (Mehendale, 2005). However, most research to date has focused on the early metabolism and toxicity after APAP overdose rather than the later recovery. Furthermore, the focus of liver regeneration research has been on partial hepatectomy (PHx) and resection rather than acute liver injury caused by drugs and other xenobiotics (Michalopoulos, 2010). More research is needed to understand liver regeneration after toxic injury so that new drug targets and biomarkers can be identified.

Phosphatidic acid (PA) is an intermediate in the synthesis of both triglycerides and membrane phospholipids. Numerous studies have demonstrated that it can also function as a lipid second messenger through activation of mechanistic target of rapamycin (mTOR) signaling (Fang et al., 2001; Foster, 2013). mTOR signaling is well known to inhibit catabolic processes and to promote cell growth and proliferation. Thus, PA may be an important mediator of liver regeneration signaling after acute injury. We hypothesized that PA levels increase after APAP overdose and enhance liver regeneration through a pathway involving mTOR. To test our hypothesis, we treated mice with a hepatotoxic dose of APAP and measured liver injury, regeneration, and PA levels over time. We also measured PA in plasma from APAP overdose patients with and without liver injury as indicated by ALT. Next, we determined the effect of overexpression of the PA phosphohydrolase (PAP) enzymes lipin 1 and lipin 2 on hepatocyte proliferation *in vitro*. Finally, we tested the effect of inhibition of PA synthesis on liver regeneration *in vivo*. Overall, our data suggest that PA is elevated during APAP hepatotoxicity due to lipin suppression, and that it promotes liver regeneration.

2. METHODS

2.1. Human subjects

Patients given a diagnosis of APAP overdose at either the University of Kansas Medical Center or the University of Arkansas for Medical Sciences were included in this study. The inclusion criteria were a physician diagnosis based on patient history, elevated serum APAP levels based on the Rumack-Matthew nomogram, abnormal liver function test results, or some combination thereof. Exclusion criteria were reasonable evidence of another major cause of liver injury. Informed consent was obtained from each patient or next of kin, and at least one blood sample was collected in an EDTA plasma or serum tube. The study protocols were approved by the Institutional Review Boards of the University of Kansas Medical Center and the University of Arkansas for Medical Sciences, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Animals

Male C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were used at 8–12 weeks age. The animals were fasted overnight to reduce variation in APAP toxicity due to differences in liver glycogen and glutathione content, then injected i.p. with either a single 300 mg/kg dose of APAP (Sigma, St. Louis, MO) dissolved in warm phosphate-buffer saline (PBS) or an equal volume of warm PBS alone. Food was returned 6 h after treatment. In two experiments, mice were also treated with 20 mg/kg FSG67 in DMSO or an equal volume of DMSO vehicle (2 mL/kg). The drug or vehicle was administered either at 2 h post-APAP to assess liver injury at 6 and 24 h, or at 24 and 48 h post-APAP to assess liver regeneration at 52 h. For all other experiments, blood and liver tissue were harvested at the time points indicated in the figures. For each mouse, liver sections were flash frozen in liquid nitrogen for later immunoblotting, PCR, or other biochemical analysis. A single liver section from each mouse was fixed in 10% formalin for histology. Blood was collected in syringes containing EDTA, and plasma was obtained by centrifugation. All animal protocols were reviewed and approved by the Institutional Care and Use Committees of the University of Arkansas for Medical Sciences and the Washington University School of Medicine.

2.3. Cell culture experiments

HepG2 cells were grown in DMEM with 10% fetal bovine serum and penicillin/streptomycin. Lipin sequences were subcloned into the Ad-track CMV vector and recombined into Ad-EASY to generate adenovirus, as described (Mitra et al., 2013). ³H-thymidine incorporation was used to assess DNA synthesis. Briefly, when cells reached approximately 70% confluence, 1 μ Ci/mL of ³H-thymidine was added to the culture medium and the cells were incubated for 4 h. Cells were then washed with PBS, and proteins were precipitated by addition of cold 10% trichloroacetic acid (TCA). After washing with 10% TCA, the protein pellet was re-solubilized by 30 min incubation in 0.3 N NaOH at room temperature and radioactivity was measured using a scintillation counter.

2.4. Clinical chemistry

Alanine aminotransferase activity was measured in plasma according to the manufacturer's instructions using a kit purchased from Teco Diagnostics (Anaheim, CA).

2.5. Histology

Formalin-fixed tissues were washed in ethanol and xylene and embedded in paraffin wax. 5–10 μ m sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for microscopy.

2.6. Western blotting

Tissues were homogenized in 25 mM HEPES buffer with 5 mM EDTA (pH 7.4), 0.1% CHAPS, and protease inhibitors, using a bead homogenizer (Qiagen, Valencia, CA). Protein concentration in the homogenates was measured using the bicinchoninic acid (BCA) assay and equal amounts of protein were loaded in each lane of a 4–20% Tris-glycine gel. After electrophoresis, proteins were transferred to PVDF membranes with 0.45 μ m pores and

blocked with 5% milk in Tris-buffered saline with 0.1% Tween 80. After incubation with the appropriate antibodies, protein bands were visualized using either the Odyssey Imaging System (LiCor Biosciences, Lincoln, NE) or x-ray film. Polyclonal Lipin 1 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Lipin 2 antibody was produced as previously described (Gropler et al., 2009). α -Tubulin clone B-5-1-2 antibody was purchased from Sigma (St. Louis, MO). Goat anti-rabbit 800 and donkey anti-mouse 800 secondary antibodies were obtained from LiCor Biosciences. All other antibodies were purchased from Cell Signaling (Boston, MA). All primary antibodies were diluted 1:1,000 in 5% milk, and all secondary antibodies were diluted 1:10,000 in 5% milk.

2.7. Phosphatidic acid measurement

For the time course studies, PA was measured in liver and plasma using LC-ESI-MS/MS. Briefly, mouse liver samples were homogenized in 400 μ L of water with the Omni Bead Ruptor 24 (Omni International, Inc., Kennesaw, GA). PA(14:0/14:0) was used as an internal standard (IS) and was added to the samples before extraction. Extraction of PA was performed by protein precipitation from 50 μ L of plasma or liver homogenate. PA analysis was performed with a Shimadzu 10A HPLC system and a Shimadzu SIL-20AC HT auto-sampler coupled to a Thermo Scientific TSQ Quantum Ultra triple-quadrupole mass spectrometer operated in SRM mode under ESI(+). Data processing was performed using Xcalibur (Thermo, Waltham, MA). For each experiment, the data were reported as peak AUC ratios relative to the IS, and then normalized to the control group mean. All PA data for each experiment were collected at the same time, so that comparison of the ratios to the within-experiment controls allowed accurate relative quantification. For the FSG67 experiments, total PA (PA+lyso-PA) was measured using a kit from Cell Biolabs (San Diego, CA). All liver PA levels were normalized to protein measured using the BCA assay before expressing as fold over control.

2.8. Statistics

Normality was tested using the Shapiro-Wilk test. For normally distributed data, Student's t-test or one-way analysis of variance (ANOVA) with Student-Newman-Keul's post-hoc test was used to compare two or more than two groups, respectively. For non-normally distributed data, the Mann-Whitney U-test or the Kruskal-Wallis test with Dunn's multiple comparisons was used. All statistical tests were performed using SigmaPlot 12.5 software (Systat, San Jose, CA). In all cases, $p < 0.05$ was considered significant.

3. RESULTS

3.1. Time course of acetaminophen-induced liver injury and recovery

To characterize the time course of acetaminophen-induced acute liver injury and recovery, mice were treated with 300 mg/kg APAP or PBS vehicle for 2, 6, 24 and 48 h and plasma ALT activity was measured. The 2 h PBS-treated mice were used as the 0 h APAP time point. Other mice were treated with PBS vehicle for 6 or 24 h for histology. Plasma ALT was significantly increased by 6 h post-APAP, and peak activity ($1,048 \pm 79$ U/L) was observed at 24 h (Fig. 1A). Activity decreased at 48 h (401 ± 133 U/L). Consistent with that, oncotic necrosis was evident in H&E-stained liver sections by 6 h post-APAP, peaked

around 24 h, and was reduced by 48 h (Fig. 1B). Also, more clear space was visible in hepatocytes at 48 h, which is suggestive of glycogen repletion due to improved hepatic function. Finally, proliferating cell nuclear antigen (PCNA) expression was detectable by 24 h and continued to increase until at least 72 h (Fig. 1C). These data demonstrate that APAP-induced liver injury in mice peaks by 24 h, and suggest that liver regeneration begins by 24 h.

3.2. Phosphatidic acid increases in liver and plasma of mice after acetaminophen overdose

We next measured PA in liver and plasma by LC-MS/MS. We observed a significant increase in total PA level by 6 h (Fig. 2A,C), which was maintained until at least 48 h in the liver (Fig. 2A). Analysis of individual PA species revealed that PA(16:0/18:2), PA(18:0/20:4) and either PA(18:0/18:2) or PA(18:1/18:1) (indistinguishable by LC-MS/MS) were significantly increased in liver tissue, and PA(16:0/18:1) showed a strong trend toward increased levels at 6, 24 and 48 h post-APAP (Fig. 2B). PA(16:0/18:1) and PA(18:0/20:4) accounted for the increase in plasma, and remained elevated until 24 h (Fig. 2D). Altogether, these data demonstrate that PA levels are increased in the liver during APAP hepatotoxicity and recovery, and may be useful as a circulating biomarker.

3.3. Phosphatidic acid increases in plasma of acetaminophen overdose patients with liver injury

To determine if PA also increases in humans with liver injury due to APAP overdose, blood was collected from patients diagnosed with APAP overdose and PA was measured in the plasma. Patient demographics and clinical laboratory test results are listed in Table 1. Similar to mice, we observed a significant increase in three PA species in overdose patients that developed evidence of acute liver injury (peak ALT ≥ 2 xULN) compared to those who did not (peak ALT < 2 xULN) (Fig. 3). We also observed a strong trend toward increased total PA in the patients with injury (Fig. 3F). Although there were no significant correlations between absolute values for PA and ALT based on Pearson correlation coefficients, receiver operating characteristic curve analysis revealed AUC values in the range of 0.80 to 0.90 for PA and peak ALT ≥ 2 xULN (Table 2).

3.4. Lipin 1 and 2 are suppressed during acetaminophen-induced liver injury and recovery

Next, we wanted to investigate the mechanism of increased PA in liver and plasma in mice. The lipin enzymes are PA phosphohydrolases that convert PA to diacylglycerol (DAG). The major lipin isoforms in the liver are lipin 1 and lipin 2. To determine if reduced lipin 1 and/or 2 expression could explain the increased PA, we measured mRNA of those enzymes. Lipin 1 mRNA significantly decreased by 12 h (0.24 ± 0.05 -fold vs. 0 h) and remained suppressed until at least 48 h (Fig. 4A). On the other hand, lipin 2 mRNA increased at 6 h post-APAP, but then showed a strong trend toward reduced expression by 24 h (Fig. 4B) like lipin 1. Western blotting for lipin 1 and lipin 2 in liver from PBS control and 6 h and 48 h post-APAP mice confirmed these results (Fig. 4C,D). These data demonstrate that lipin 1 expression is decreased by 12 h after APAP overdose, and that both lipin 1 and 2 are decreased at later time points, during recovery.

Because PA levels increased in the liver at 6 h post-APAP, but lipin expression did not decrease until 12 h or later, we wondered if the early rise in PA could be due to suppression of lipin activity. To test that possibility, we measured lipin-specific Mg^{2+} -dependent PAP activity in the liver at 0, 6 and 24 h post-APAP. Importantly, we observed a decrease in lipin activity by 6 h (Fig. 4E,F). These data suggest that the elevated PA levels early after APAP overdose are due to suppression of lipin activity, rather than expression. Thus, the early reduction in lipin activity was likely due to post-translational modifications, as there was no change in lipin 1 protein at that time point and lipin 2 protein even increased.

3.5. Expression of other phosphatidic acid metabolism genes after acetaminophen treatment

To determine if changes in expression of other PA-metabolizing genes could also contribute to the observed changes in liver PA levels, we measured mRNA for the major liver isoforms of glycerol-3-phosphate acyltransferase (Gpat), 1-acylglycerol-3-phosphateacyl-transferase (Agpat), phospholipase D (Pld), and diacylglycerol kinase (Dagk) (Fig. 5). While lipin 1 and lipin 2 are important for metabolism of PA, these other enzymes are important for synthesis of PA. Although we did find a transient decrease in mRNA for Agpat1 at 6 h (Fig. 5B) and for Dagk α at 24 h (Fig. 5D), there were no long-term changes in expression of any of these genes and none increased (Fig. 5). Thus, suppression of lipin enzymes appears to be the primary mechanism of increased PA levels in the liver during APAP-induced liver injury and recovery.

3.6. Lipin overexpression reduces hepatocyte proliferation *in vitro*

We next wanted to test the effect of PA on hepatocyte proliferation. It is thought that direct treatment of cultured cells with PA causes off-target effects due to 1) conversion of PA to lysophosphatidic acid (lyso-PA) by phospholipase A₂ and other PA-metabolizing enzymes often present in cell culture medium, and 2) interactions with cell surface receptors. Thus, to test the effect of PA on cell proliferation, indirect methods of PA modulation must be used. We overexpressed lipin 1 and lipin 2 individually in the human hepatocyte cell line HepG2 to reduce PA content and measured DNA synthesis by ³H-thymidine incorporation. Importantly, overexpression of either lipin enzyme (Fig. 6A) resulted in reduced DNA synthesis (Fig. 6B). These data indicate that PA promotes hepatocyte proliferation.

3.7. Inhibition of PA synthesis reduces liver regeneration *in vivo*

To determine if reducing PA content in the liver can **affect liver injury** or blunt hepatocyte proliferation *in vivo*, we treated mice with APAP followed by the Gpat inhibitor FSG67 or DMSO vehicle control at 2, 24, and 48 h post-APAP. APAP metabolism is rapid, and complete by 2 h post-APAP (McGill et al., 2013), so this post-treatment regimen is not expected to affect APAP bioactivation. We measured ALT at 24, 48 h, and 52 h post-APAP, and PCNA protein at 52 h. There was no difference in ALT between the vehicle and FSG67 treated mice at any time point (Fig. 7A), which confirmed that the post-treatment regimen did not affect APAP bioactivation. However, PCNA was significantly decreased at 52 h in liver tissue from the FSG67-treated animals (Fig. 7B). To avoid the potential for any unexpected effects of vehicle or FSG67 on the early liver injury, we also repeated the

experiment with only the 24 and 48 h post-APAP treatments (Fig. 7C,D). Again, FSG67 reduced PCNA levels at 52 h post-APAP (Fig. 7D). We also observed a significant increase in ALT in the FSG67-treated mice. The reason for the difference in ALT results at 52 h between the two experiments is not clear, but an increase in ALT is consistent with reduced recovery. Finally, we measured PA in all of the FSG67 and vehicle-treated mice at 52 h post-APAP to ensure that the drug did in fact reduce PA content in the liver. As expected, PA values were significantly lower in the FSG67-treated mice (Fig. 7E). Together, these data demonstrate that Gpat inhibition reduces hepatocyte proliferation *in vivo*. These results are consistent with a role for PA in liver regeneration after acute injury.

3.8. Time course of mTOR phosphorylation after acetaminophen overdose

Finally, to begin to test if the increased PA activates mTOR signaling after APAP overdose, we measured mTOR phosphorylation by immunoblot. Surprisingly, p-mTOR was increased by 2 h post-APAP, before the increase in PA (Fig. 8). p-mTOR levels were then maintained until at least 48 h (Fig. 8). It is possible that some initial stimulus activates mTOR signaling at early time points after APAP overdose and PA then maintains those levels. Additional studies are needed to address the role of PA in late mTOR activation after APAP overdose.

4. DISCUSSION

Little is known about the mechanisms of liver regeneration after APAP overdose. Most studies of liver regeneration have relied on the partial hepatectomy model (PHx) in which 2/3 of the liver is removed and the remaining lobes grow to restore functional mass. It is clear from those studies that growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor receptor (EGFR) ligands are necessary for liver regeneration (Paranjpe et al., 2016; Michalopoulos, 2017), that various cytokines help to prime hepatocytes for growth (Fausto et al., 2006; Michalopoulos, 2017), and that large changes in nutrient homeostasis resulting from the PHx can also affect regeneration (Huang and Rudnick, 2014; Huang et al., 2016). The details of those signaling pathways have been studied for decades, but few groups have attempted to investigate the mechanisms of liver regeneration after drug-induced liver injury. It is known that interleukin-6 (IL-6) deficiency delays liver regeneration after APAP overdose in mice (James et al., 2003). More recent data have also demonstrated that EGFR and the HGF receptor c-Met are activated in APAP hepatotoxicity, and that inhibition of EGFR reduces hepatocyte proliferation (Bhushan et al., 2014; 2017). Similar effects have been described in mice treated with other acute hepatotoxicants (Phaneuf et al., 2004; Scheving et al., 2015). However, no effort has been made to study the effects of metabolic changes during APAP hepatotoxicity on liver regeneration, even though defects in mitochondrial respiration and lipid metabolism after APAP overdose have been described in both mice and humans in numerous reports (Meyers et al., 1988; Coen et al., 2003; Chen et al., 2009; McGill et al., 2014b; Bhattacharyya et al., 2014).

We observed an increase in the lipid PA in both plasma and liver tissue from mice during APAP hepatotoxicity. The increase in PA coincided with a decrease in expression of the enzymes lipin 1 and lipin 2, and with reduced lipin-specific PAP activity, suggesting that the

mechanism of PA elevation was suppression of lipin enzymes. The fact that lipin activity decreased before the decrease in expression also suggests that the activity is regulated by post-translational modification early after APAP overdose. It is already known that mTOR-mediated phosphorylation deactivates lipin 1 (Harris et al., 2007; Eaton et al., 2013). Future experiments will determine whether this or other post-translational modifications occur after APAP overdose. To determine if PA levels affect hepatocyte proliferation and liver regeneration, we overexpressed lipin 1 or lipin 2 in HepG2 cells. Importantly, transduction of either enzyme reduced DNA synthesis. Finally, we found that the PA synthesis inhibitor FSG67 reduced an important marker of liver regeneration *in vivo*. Together, the data are consistent with our hypothesis that PA enhances liver regeneration after APAP hepatotoxicity.

It has been known for decades that PA is mitogenic. As early as 30 years ago, it was shown that treatment of cultured cells with PA can induce DNA synthesis (Siegmann, 1987). Recent data suggest that the pro-proliferative effects of PA occur through mTOR. Fang et al. (2001) were the first to demonstrate that PA activates mTOR signaling in cultured cells. Although it was later demonstrated that exogenous PA is converted to lyso-PA by the action of PLA₂ in the culture medium and that lyso-PA is actually responsible for the effect on mTOR in an ERK-dependent manner (Winter et al., 2010), numerous other studies have also revealed a direct interaction of PA with mTOR (Fang et al., 2001; Veverka et al., 2008; You et al., 2012) and it has been demonstrated that PA activates mTOR in cells through both ERK-dependent and -independent mechanisms (You et al., 2012). Finally, treatment with di-8:0 PA activates mTOR in cultured cells (Yoon et al., 2011; 2015), and di-8:0 PA has biological actions in cell culture that 8:0 lyso-PA does not have (Fischer et al., 2001). Thus, the weight of the evidence supports a direct effect of intracellular PA on mTOR, though indirect effects through extracellular lyso-PA likely also occur. Importantly, mTOR signaling enhances liver regeneration. Treatment with rapamycin after PHx inhibits cell proliferation in the liver (Palmes et al., 2008; Espeillac et al., 2011; Fouraschen et al., 2013), and genetic deficiency of the mTOR target S6K1 has a similar effect (Espeillac et al., 2011). Moreover, chronic mTOR activation is carcinogenic in the liver (Menon et al., 2011), also demonstrating that it promotes hepatocyte proliferation. Interestingly, we observed increased phosphorylation of mTOR as early as 2 h after APAP overdose, and that effect was sustained until at least 48 h. Therefore, it is possible that the initial mTOR activation leads to the increase in PA in the liver through inhibition of lipin 1, and the PA then maintains mTOR activation at later time points resulting in a feed-forward loop that enhances regeneration. Additional studies are needed to test that possibility and to investigate the mechanism of the early mTOR activation and lipin suppression.

The fact that the Gpat inhibitor FSG67 reduced PCNA expression after APAP hepatotoxicity is consistent with a role for PA in liver regeneration. However, Gpat inhibition would also be expected to reduce lyso-PA. Thus, we cannot rule out the possibility that lyso-PA is also important. Our *in vitro* experiments with overexpression of lipin 1 and lipin 2 in HepG2 cells may be more specific for PA, but we did not demonstrate that experimentally. While a more direct approach to test the effect of PA on liver regeneration or hepatocyte proliferation would be to treat with exogenous PA, we did not do that for two reasons. First, the major effect that we observed in our experiments was an increase in intrahepatic PA, and it is well

known that the mechanisms of intracellular and extracellular PA signaling differ (Shad et al., 2015). Although both activate mTOR, extracellular PA activates multiple additional signaling pathways that could cause off-target effects (Shad et al., 2015). For example, as discussed above, extracellular PA is likely converted to lyso-PA in plasma (Winter et al., 2010), and lyso-PA activates cell surface receptors such as the lysophosphatidic acid receptor 1 (Lpar1), a G protein-coupled receptor (Winter et al., 2010; Shad et al., 2015). PA itself likely also binds to some extracellular receptors. Second, PA was already increased by APAP overdose. Thus, we believed that the best approach would be to prevent or reduce the accumulation of PA, rather than trying to enhance it.

Our discovery that PA is increased in plasma from both mice and humans suggests that it could serve as a biomarker of liver regeneration. Regeneration is generally thought to be critical for survival in ALF in humans. Several studies support that idea. Indices of proliferative activity in liver sections correlate with liver function and patient outcome (Kayano et al., 1992; Katoonizadeh et al., 2006). It is also known that α -fetoprotein (AFP), a marker of hepatocyte immaturity and proliferation, is a strong predictor of survival after APAP overdose (Schmidt and Dalhoff, 2005), although its utility is limited by the fact that its levels do not increase until after the liver injury has occurred. Finally, a recent meta-analysis of clinical trials also demonstrated that administration of granulocyte-colony stimulating factor (G-CSF), which is known to accelerate liver repair in animal models by promoting migration of stem cells into the damaged liver, reduces mortality in acute-on-chronic liver failure patients (Chavez-Tapia et al., 2015). Due to the apparent importance of regeneration for survival, it seems likely that biomarkers that reflect regenerative signaling could be useful for prediction of patient outcome. Also, the FDA and European Medicines Agency have recently expressed support for development of biomarkers that can differentiate between patients who are likely to develop true, severe liver injury, and those who will adapt and survive (EMA, 2016; Teschke et al., 2017). It is possible that PA and similar lipid metabolites could be used for that purpose.

One potential problem with the use of PA as a circulating biomarker of regeneration and survival revealed by our data is that PA was not elevated compared to the control group for each individual patient with liver injury. Because most of the patients in our study were survivors, that suggests that PA is not elevated in all survivors and therefore has poor sensitivity for survival. However, our data are limited by the fact that we only measured PA at one time point for each patient, and the time points were not standardized to either the time of presentation or the reported time of APAP overdose. Thus, although there was considerable overlap in values for most PA species between the two groups, that overlap could simply be due to variation in sample timing. It is also possible that PA has high intra-individual variation, such that a baseline value would need to be taken at the time of patient presentation in order to be clinically useful, similar to the use of cardiac troponins to diagnose myocardial infarction. Clearly, additional studies are needed to determine the clinical utility of circulating PA.

Importantly, while this is the first study to explore the role of hepatic lipin enzymes and PA in liver regeneration, we are not the first to investigate the role of lipin enzymes in liver injury. The effects of lipin 1 have been thoroughly investigated in mouse models of alcoholic

liver disease (ALD). In contrast to our results, Hu et al. (2012) reported that ethanol induces expression of lipin 1 in the liver. Induction of lipin 1 appears to be protective in ALD; hepatic lipin 1 deficiency actually enhances alcoholic steatosis and inflammation in mice (Hu et al., 2013). Interestingly, those effects are probably due to loss of the co-regulatory transcription factor function of lipin 1 within hepatocytes (Hu et al., 2013). It is not yet clear what role that function of lipin 1 has in APAP toxicity, but it is interesting to note that mitochondrial biogenesis is important in liver regeneration after APAP overdose (Du et al., 2017) and lipin 1 directly interacts with the mitochondrial biogenesis mediator PGC-1 α to amplify its function (Finck et al., 2006).

The effects of lipin 1 in liver injury are not limited to hepatic lipin 1. Surprisingly, myeloid-specific lipin 1 deficiency has the opposite effect of hepatic deficiency on ALD, causing reduced inflammation and liver injury (Wang et al., 2016). That finding may also have implications for APAP hepatotoxicity, as inflammation has been thought to play a role in both injury and regeneration after APAP overdose. The importance of inflammation in APAP hepatotoxicity is highly controversial (Woolbright and Jaeschke, 2017a, 2017b; Kennon-McGill and McGill, 2018) and experiments with myeloid-specific lipin 1 deficient or transgenic mice could add clarity. Another study revealed that overexpression of lipin 1 in adipose tissue is also protective against ALD (Zhang et al., 2018). Clearly, future studies should examine the role of extrahepatic lipin enzymes in APAP hepatotoxicity.

Taken together, our data demonstrate that PA is increased in liver and plasma during APAP hepatotoxicity, and may enhance liver regeneration and therefore recovery. Our data have broad implications for all forms of liver injury and disease that involve hepatocyte proliferation, including cancer, hepatic resection, hypoxic hepatitis, and cirrhosis. Our future studies will focus on the regulation of lipin enzymes during liver injury, and will explore the roles of mTOR and PA in liver regeneration after APAP hepatotoxicity in much greater detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALF acute liver failure

ALT	alanine aminotransferase
APAP	acetaminophen
Gpat	glycerol-3-phosphate acyltransferase
LC-ESI-MS/MS	liquid chromatography with electrospray ionization and tandem triple quadrupole mass spectrometry
lyso-PA	lysophosphatidic acid
mTOR	mechanistic target of rapamycin
NAC	N-acetylcysteine
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase
PCNA	proliferating cell nuclear antigen
PHx	partial hepatectomy
ULN	upper limit of normal

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HIGHLIGHTS

- Phosphatidic acid increases in liver and circulation in mice after APAP overdose
- Phosphatidic acid increases in circulation of humans with APAP hepatotoxicity
- Reducing phosphatidic acid content in the liver blunts liver regeneration
- Phosphatidic acid may affect liver regeneration through mTOR signaling

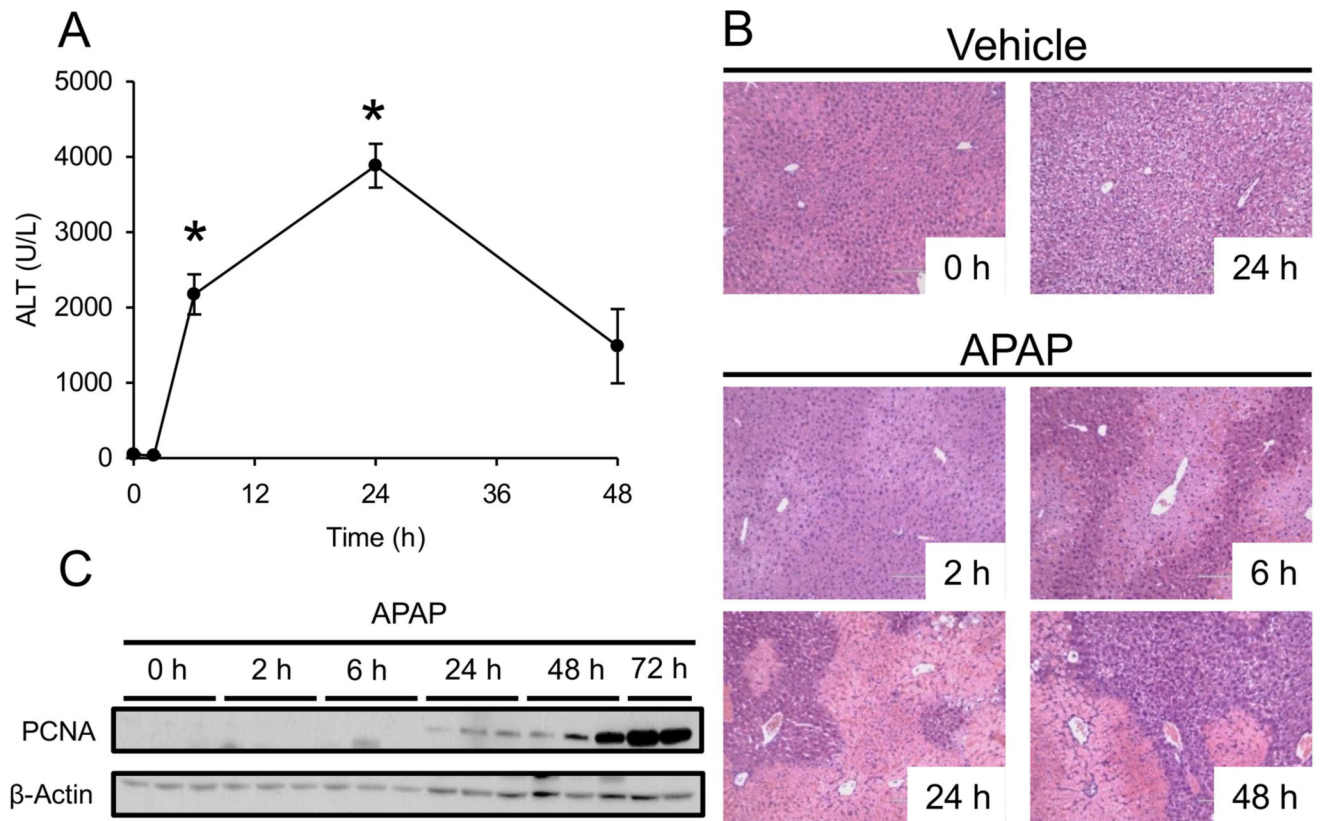


Figure 1. Time course of liver injury and regeneration after acetaminophen overdose
 Male C57Bl/6J mice were treated with 300 mg/kg APAP or PBS vehicle and blood and liver tissue were collected at the indicated time points. (A) Plasma ALT activity. (B) H&E-stained liver sections. (C) Proliferating cell nuclear antigen (PCNA) immunoblot. Data are expressed as mean \pm SEM for n = 3–4. *p<0.05 vs. 0 h and Veh.

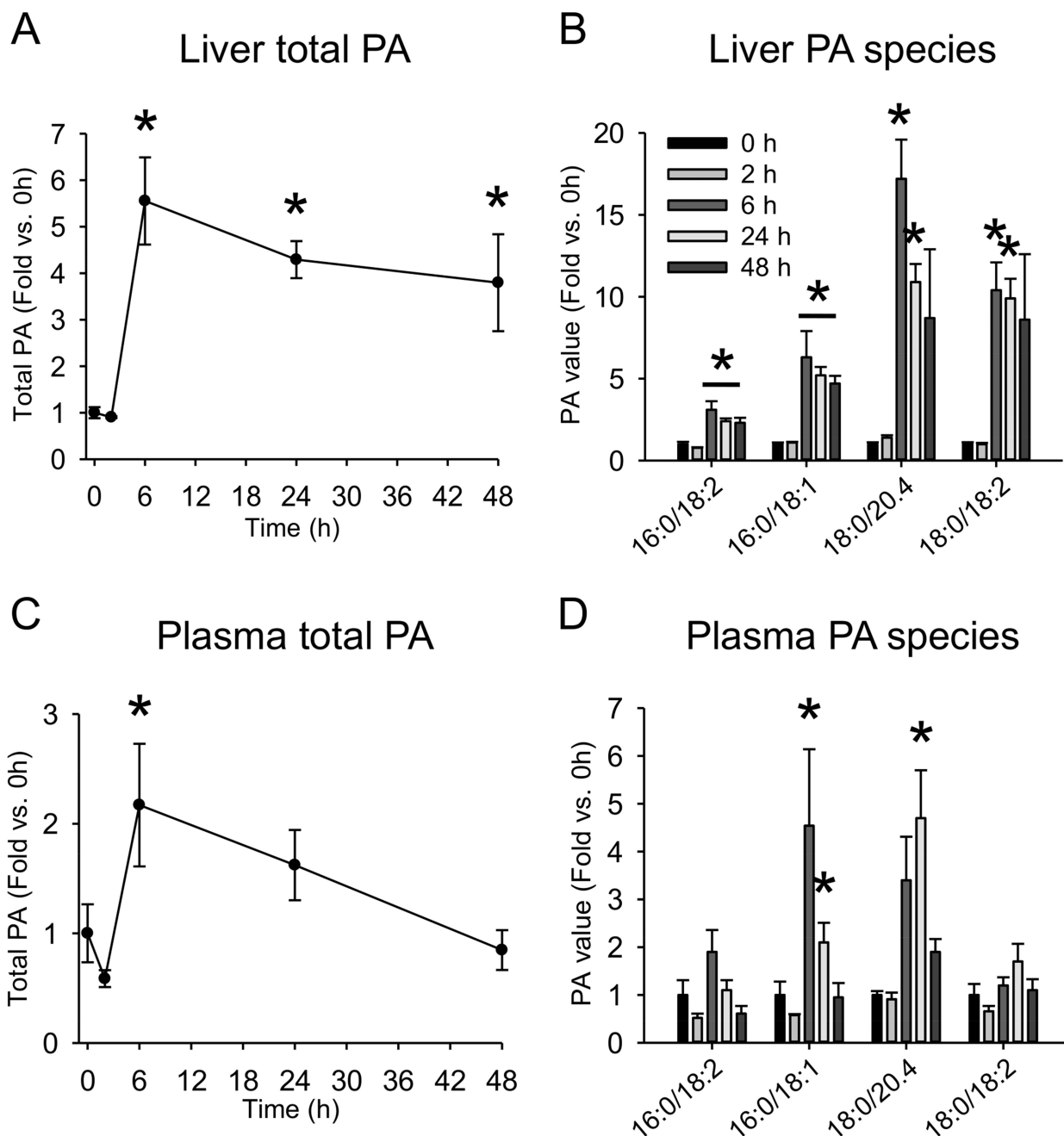


Figure 2. Time course of phosphatidic acid after acetaminophen overdose

Male C57Bl/6J mice were treated with 300 mg/kg APAP or PBS vehicle and blood and liver tissue were collected at the indicated time points. (A) Total liver phosphatidic acid (PA) content. (B) Individual PA species in the liver. (C) Total plasma PA content. (D) Individual PA species in the plasma. Data are expressed as mean±SEM for n = 3. *p<0.05 vs. 0 h and Veh.

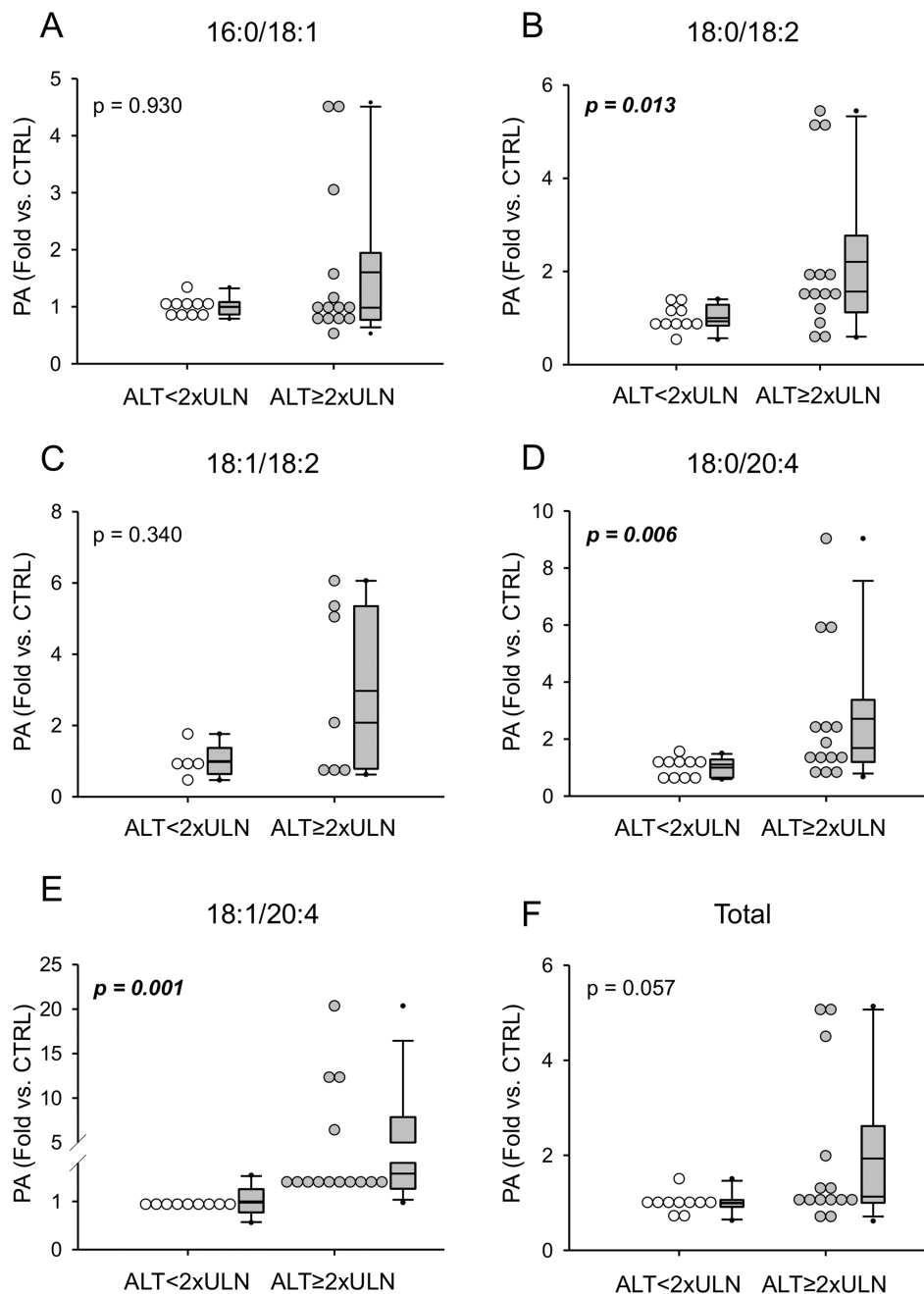


Figure 3. Phosphatidic acid values in acetaminophen overdose patients

Phosphatidic acid species were measured in plasma or serum from acetaminophen overdose patients and compared between patients that did not develop liver injury (ALT less than 2x the upper limit of normal [ULN]) and those that did (ALT ≥ 2xULN). (A) 16:0/18:1 PA. (B) 18:0/18:2 PA. (C) 18:1/18:2 PA. (D) 18:0/20:4 PA. (E) 18:1/20:4 PA. (F) Total PA. Data are expressed as fold over ALT < 2xULN patients. Whiskers show the 5th to 95th percentile, box plots show the 25th to 75th percentile, and lines show the median and mean.

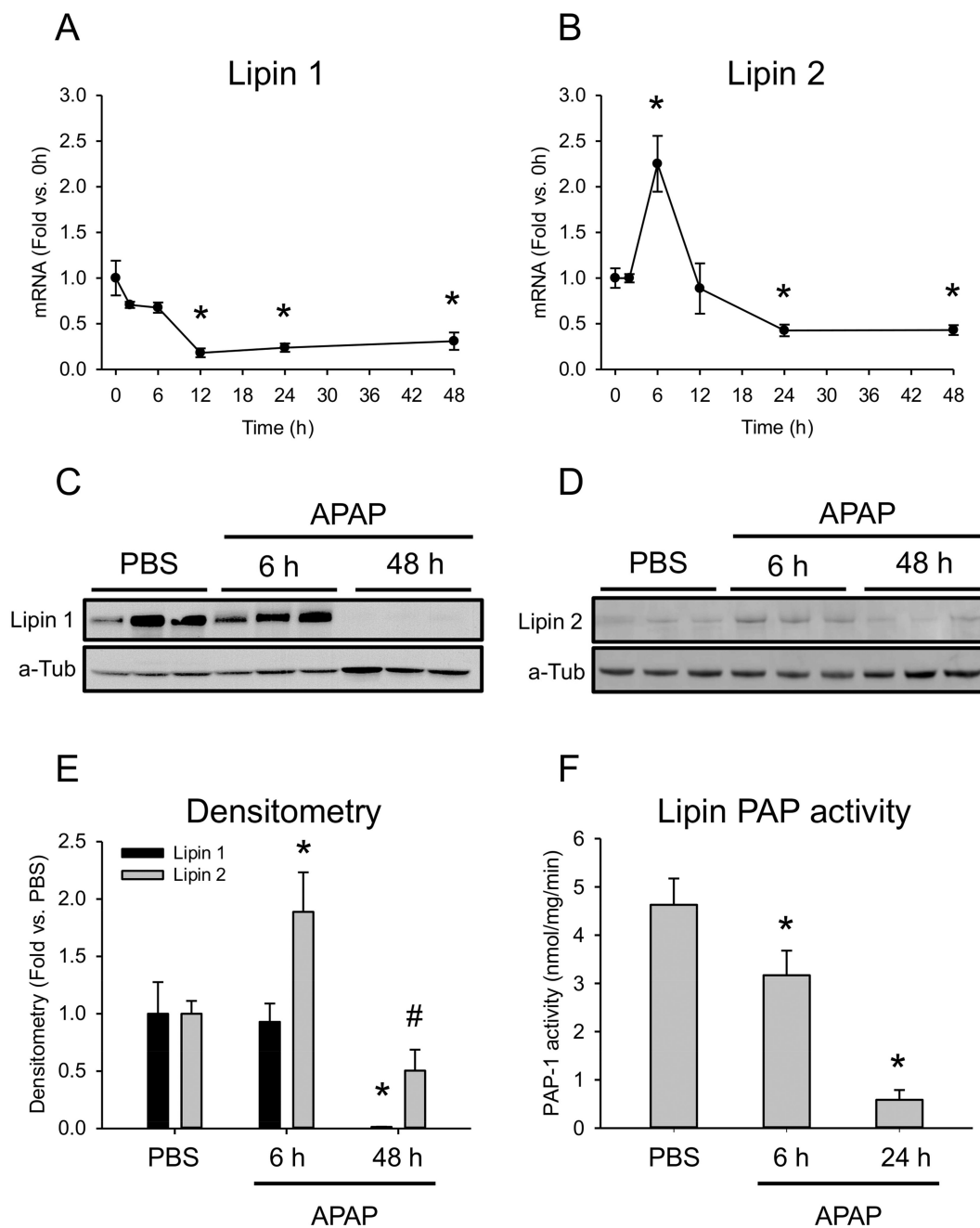


Figure 4. Time course of lipin 1 and 2 expression and activity after APAP overdose

Male C57Bl/6J mice were treated with 300 mg/kg APAP or PBS vehicle and liver tissue was collected at the indicated time points. (A) Lipin 1 mRNA in the liver. (B) Lipin 2 mRNA in the liver. (C) Liver lipin 1 immunoblot. (D) Liver lipin 2 immunoblot. (E) Densitometry for lipin 1 and lipin 2 immunoblots. (F) Lipin-specific Mg^{2+} -dependent PAP activity in the liver. Data are expressed as mean \pm SEM for n=3–6. *p<0.05 vs. PBS or 0 h. #p = 0.1 vs. PBS.

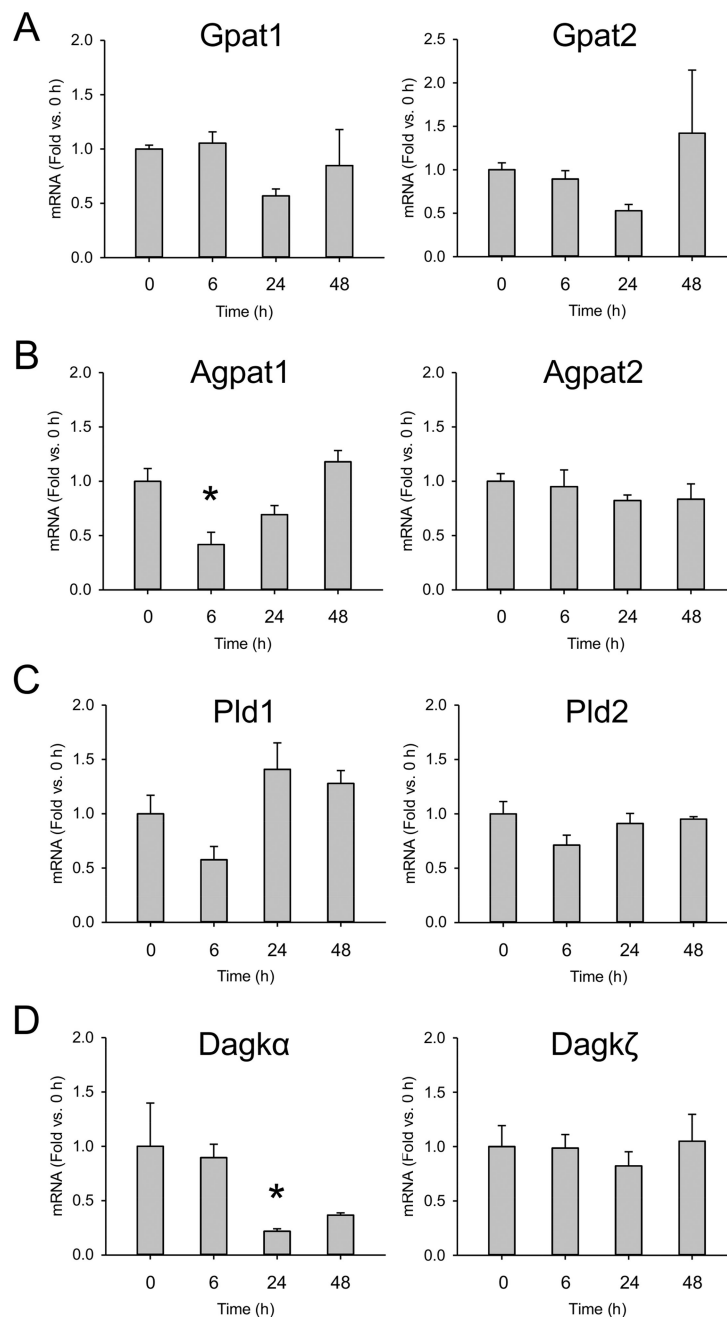


Figure 5. Expression of genes for phosphatidic acid synthesis enzymes

Male C57Bl/6J mice were treated with 300 mg/kg APAP or PBS vehicle control and liver tissue was collected at the indicated time points. (A) mRNA for the major isoforms of glycerol-3-phosphate acyltransferase (Gpat) in the liver. (B) mRNA for the major isoforms of acylglycerol phosphate acyltransferase (Agpat) in the liver. (C) mRNA for the major isoforms of phospholipase D (Pld) in the liver. (D) mRNA for the major isoforms of diacylglycerol kinase (Dagk) in the liver. Data are expressed as mean \pm SEM for n = 3–6. *p<0.05 vs. vehicle control (CTRL).

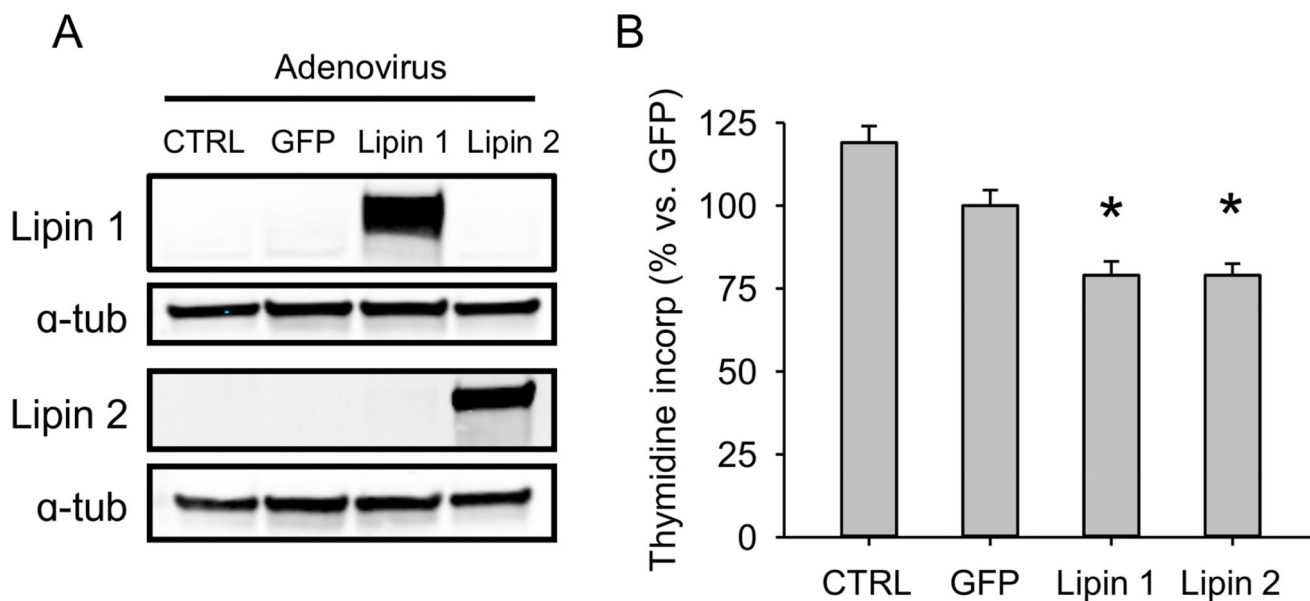


Figure 6. Effect of lipin enzymes on DNA synthesis *in vitro*

HepG2 cells were untreated or transduced to express GFP, lipin 1, or lipin 2, and DNA synthesis was measured by ^3H -thymidine incorporation. (A) Immunoblots for lipin 1 and 2 after adenovirus transduction. (B) Thymidine incorporation. Values are compared to the GFP transduction control. Data are expressed as mean \pm SEM for $n = 4$ independent experiments, each performed in triplicate. * $p < 0.05$ vs. GFP control.

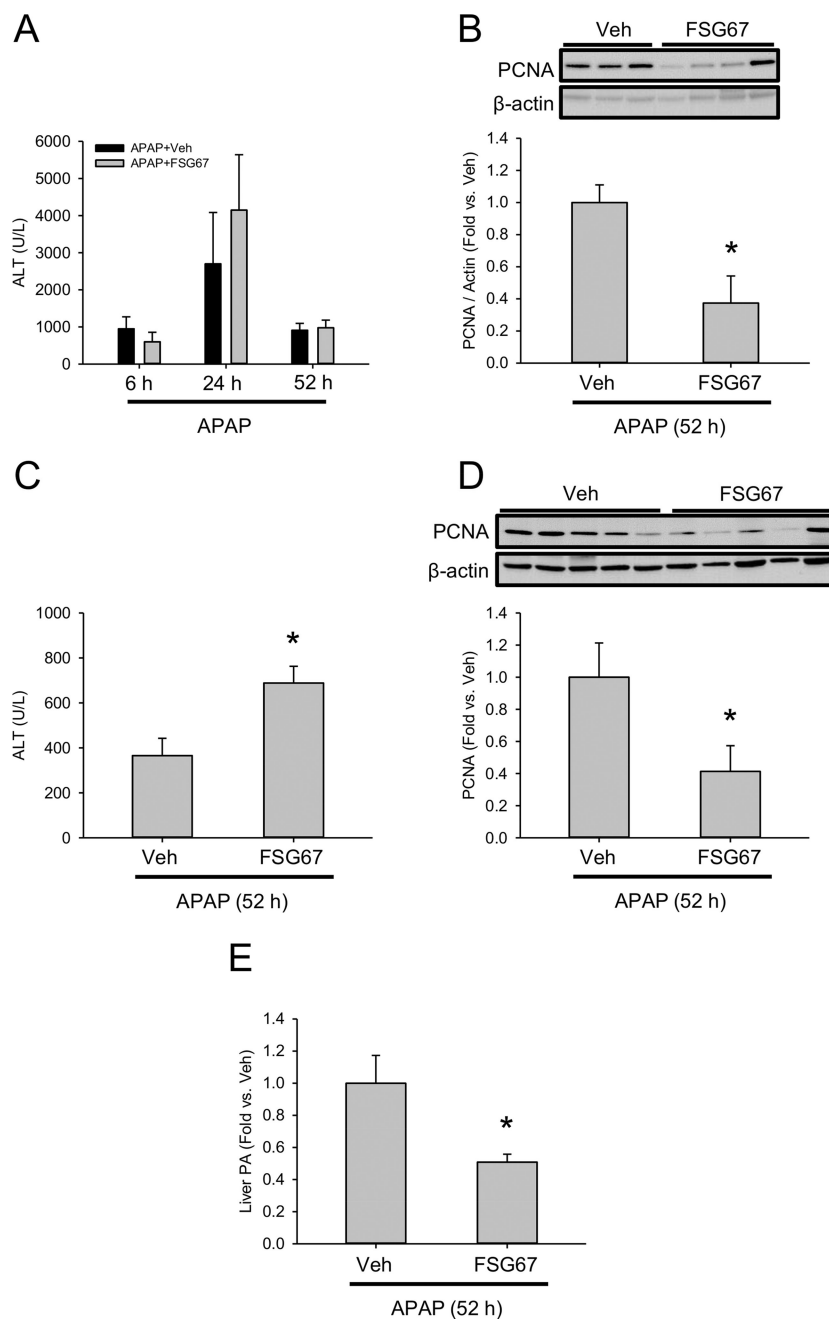


Figure 7. Effect of phosphatidic acid synthesis inhibition on liver injury and regeneration after APAP overdose

Male C57Bl/6J mice were treated with 300 mg/kg APAP or PBS vehicle, followed by 20 mg/kg FSG67 at either 2, 24, and 48 h post-APAP (A,B) or at 24 and 48 h post-APAP (C,D). Blood and liver tissue were collected at the indicated time points. (A) Plasma ALT at 6, 24 and 52 h post-APAP. (B) Immunoblot for liver PCNA and associated densitometry at 52 h post-APAP. (C) Plasma ALT at 52 h post-APAP. (D) Immunoblot for liver PCNA and associated densitometry at 52 h post-APAP. (E) Liver PA content at 52 h post-APAP. Data are expressed as mean \pm SEM for n = 3–5. *p<0.05 vs. PBS treatment. #p<0.05 vs. Veh.

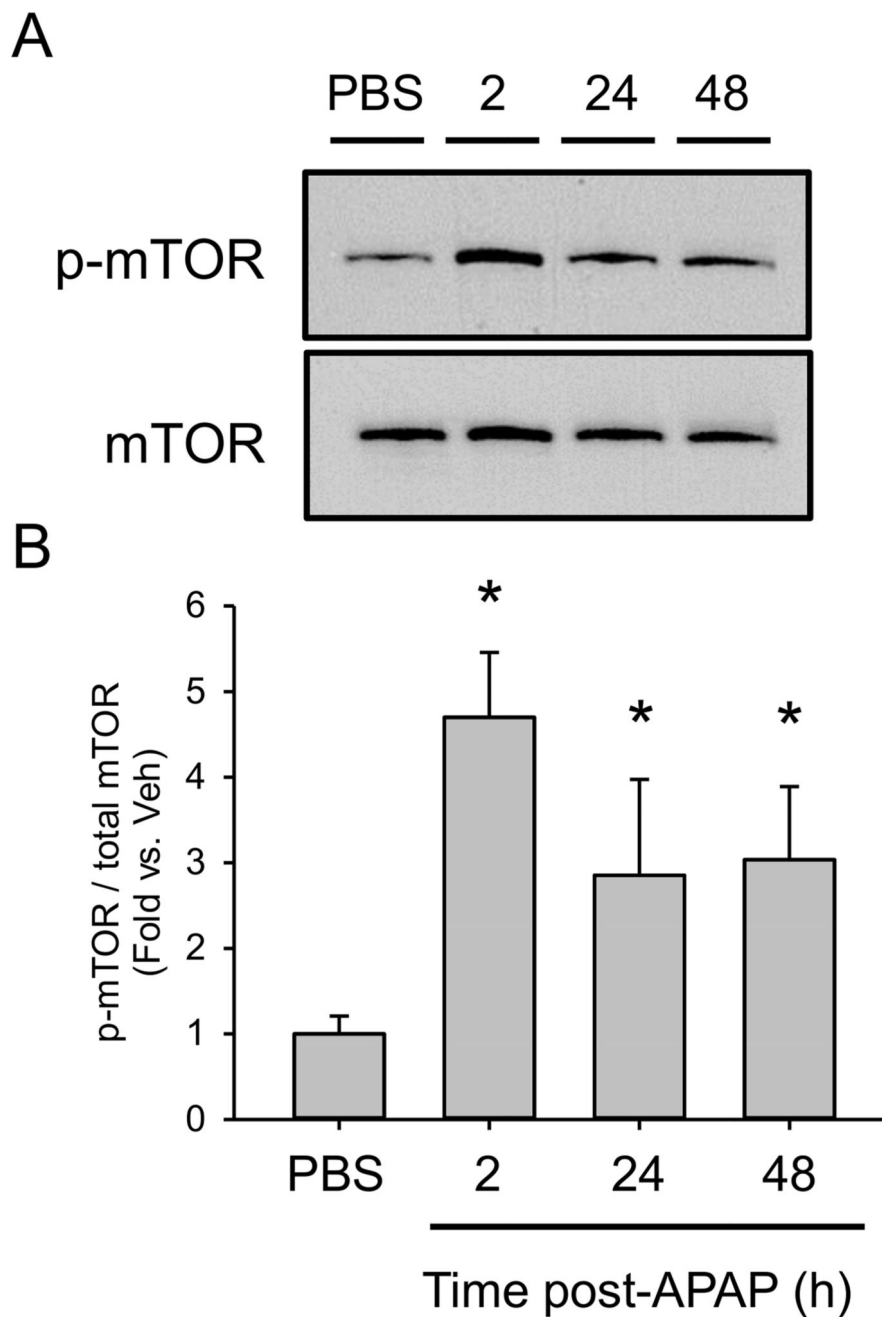


Figure 8. Phosphorylation of mTOR after acetaminophen treatment

Male C57Bl/6J mice were treated with 300 mg/kg APAP or saline vehicle and liver tissue was collected at the indicated time points. (A) Immunoblots for p-mTOR and total mTOR from pooled samples. (B) Densitometry for phosphorylated mTOR (p-mTOR) and total mTOR from non-pooled samples. Data are expressed as mean \pm SEM for n = 3. *p<0.05 vs. Veh.

Table 1

Patient demographics and clinical laboratory test results.

	ALT <2xULN	ALT 2xULN
N	10	13
Age (yr) (median, range)	41, 18–80	42, 19–63
Sex (% F)	78	69
Peak ALT (median, range)	43, 29–69	3895, 800–5365
Peak PT (s) (median, range) *	16, 11–23	43, 14–165
Peak MELD (median, range) *	7, 6–16	22, 8–46
Outcome (% survival)	100	92

* When available.

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Table 2

Receiver operating characteristic curve results for PA and acute liver injury.

PA species	AUC	Cutoff (Fold)	Sensitivity (%)	Specificity (%)
18:0/18:2	0.81	1.43	71	100
18:0/20:4	0.84	1.29	71	90
18:1/20:4	0.89	1.41	71	90
Total	0.74	1.10	57	90

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