Blocking mitochondrial alanine and pyruvate metabolism in hepatocytes worsens acetaminophen-induced liver injury in mice

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Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; APAP, acetaminophen; CKO, combined ALT2/MPC knockout; JNK, c-Jun N-terminal kinase; KO, knockout; LDH, lactate dehydrogenase; MPC, mitochondrial pyruvate carrier; NAPQI, N-acetyl-p-benzoquinone imine; P450, cytochrome P450; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; TCA, tricarboxylic acid;

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ABSTRACT

Background and Aims: Acetaminophen (APAP) overdose causes mitochondrial damage and acute liver injury. Alanine and pyruvate are important substrates for the TCA cycle and increasing evidence points to a protective role for mitochondrial intermediary metabolism in APAP hepatotoxicity. We hypothesized that suppressing hepatic alanine and pyruvate metabolism by blocking alanine transaminase (ALT) and/or the mitochondrial pyruvate carrier (MPC) would impair mitochondrial metabolism in APAP toxicity and therefore exacerbate APAP-induced injury.

Methods: We treated wild-type mice with the ALT inhibitor β-chloro-*l*-alanine (BCLA) before a toxic dose of APAP (300 mg/kg) and measured liver injury (serum ALT and lactate dehydrogenase [LDH], histology) and other parameters. We also generated liver-specific ALT2 knockout (KO) mice, mitochondrial pyruvate carrier 2 (MPC) KO mice, and combined ALT2/MPC KO (CKO) mice, and compared injury, APAP-protein adducts, glutathione, c-Jun N-terminal kinase activation, *Cyp2e1* expression, and serum metabolomics among genotypes.

Results: BCLA reduced hepatic ALT activity up to 65% but did not affect injury. ALT2 deletion also had no effect on APAP hepatotoxicity. Similarly, MPC deletion alone had no effect. However, combined ALT2 and MPC loss significantly worsened injury ($p = 0.005$ for LDH for WT vs. CKO; $p = 0.07$ for % necrosis), increased plasma pyruvate content ($p = 0.032$), altered the plasma abundance of several amino acids, and decreased basal hepatic glutathione ($p = 0.053$). Importantly, the exacerbation of injury in CKO mice was not associated with increased APAP-protein adducts.

Conclusions: The MPC and ALT systems are critical redundant mechanisms to conserve mitochondrial metabolism and limit APAP toxicity. ALT activity is more than a passive biomarker of injury in this context.

INTRODUCTION

Acetaminophen (APAP) is one of the most widely used antipyretic and analgesic drugs in the world. In a cross-sectional survey of the US population, >20% of both adults and children reported use of APAP at least once in the preceding seven days[1,2] and similar data have been reported for other countries[3–5]. However, APAP overdose can cause serious liver injury, sometimes leading to acute liver failure (ALF) and death[6].

Mitochondrial dysfunction is the central driver of APAP hepatotoxicity[7–9]. Cytochrome P450 enzymes (P450s), particularly CYP2E1, convert APAP to the reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI)[7], which depletes glutathione and then preferentially binds to mitochondrial proteins[10,11]. The mitochondrial protein alkylation leads to reduced mitochondrial respiration[12] and increased production of reactive oxygen species[13,14]. The resulting oxidative stress then promotes phosphorylation and activation of the c-Jun N-terminal kinases (JNK) 1/2[15,16], which translocate to mitochondria and further reduce mitochondrial respiration via SHP1-mediated inhibition of Src[7,17]. Eventually, the mitochondrial permeability transition occurs[18], mitochondria swell and their membranes rupture[19], and mitochondrial proteins are released into the cytosol. Among the proteins released, certain endonucleases can cleave nuclear DNA[20]. The damaged hepatocytes ultimately die by necrosis[9,21,22]. Not surprisingly then, mounting evidence indicates that conserving mitochondrial flux of pyruvate is protective in APAP toxicity by supporting mitochondrial metabolism[22–24]. Specifically, it was recently reported that mice deficient in pyruvate dehydrogenase kinase 4 ($Pdk4^{-/-}$) have constitutive activation of pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA for progression into the TCA cycle, and exhibit reduced liver injury after APAP overdose[24].

Most mitochondrial pyruvate is generated in the cytosol as a product of glycolysis and taken up via the mitochondrial pyruvate carrier (MPC) complex in the inner mitochondrial membrane[25] (Fig. 1). However, pyruvate can also be generated through other reactions, including transamination of alanine catalyzed by the alanine aminotransferases (ALT) 1 and 2[25]. ALT1 generates pyruvate from alanine in the cytosol for uptake via the MPC similar to glycolysis, while ALT2 resides in the mitochondrial matrix and converts alanine to pyruvate directly within mitochondria. Either way, once in the mitochondrial matrix, pyruvate can be oxidized by PDH or carboxylated by pyruvate carboxylase (PC) to feed the TCA cycle and studies have demonstrated that both these anaplerotic and cataplerotic pathways are critical for the overall maintenance of TCA cycle flux and metabolic homeostasis[26,27].

ALT activity has been measured in serum as a biomarker of liver injury since the 1950s[28]. Though its physiological functions are rarely considered in the context of liver disease, we recently demonstrated a role for ALT2 in the development of hyperglycemia in diabetic mice[29]. Here, we hypothesized that ALT2 is beneficial in APAP hepatotoxicity due to its function as an intra-mitochondrial source of pyruvate and because of the importance of maintaining mitochondrial metabolism during APAP-induced injury. We tested this hypothesis using both pharmacologic and genetic approaches. We also explored the roles of combined loss of MPC and/or ALT activity on the response to APAP. These studies unveiled functional redundancy between the ALT and MPC proteins, but also demonstrated that complete loss of pyruvate-alanine cycling exacerbates APAP toxicity in mice.

METHODS

Animals. Male C57BL/6J mice between the ages of 7 and 8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice that lack ALT2 were generated by the Knockout Mouse Project Repository (KOMP) (project ID CSD24977) using the "knockout first" approach wherein the LacZ/Neo cassette constitutively blocks expression of the gene until it is removed by *flp* recombinase. This construct also contained exon 4 of the *Gpt2* gene, which encodes ALT2, flanked by LoxP sites. We purchased frozen sperm from *Gpt2* germline heterozygous mice from KOMP and established a colony of mice by in vitro fertilization. C57BL/6 mouse embryonic stem cells containing a ''knockout first'' allele of *Mpc2* were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM) (International Knockout Mouse Consortium [IKMC] project 89918, catalog no. MAE-1900). This construct contains a targeted allele of *Mpc2* containing inserted cassettes for promoter-driven LacZ and Neo cDNAs flanked by *frt* sites upstream of exon 3 of the *Mpc2* gene, which is flanked by LoxP sites. Propagated ESCs were injected into developing embryos and then implanted into pseudopregnant females. Chimeric offspring were mated to establish germline transmission.

The *Mpc2* and *Gpt2* transgenic lines were then independently intercrossed with mice expressing *flp* recombinase in a global manner (B6.Cg-Tg(ACTFLPe)9205Dym/J mice; Jackson Laboratory stock number: 005703) to remove the LacZ and Neo cassettes and generate floxed mice. Floxed mice were then crossed with transgenic mice expressing *Cre* under control of the albumin promoter (B6.Cg-Speer6-ps1Tg(Alb-cre)21Mgn/J; Jackson Laboratory stock number: 003574) to create liver-specific knockout mice that have been previously described[29,30]. Combined liver-specific ALT2/MPC knockout (CKO) mice were then generated by intercrossing

the two liver-specific single gene knockouts. Littermate mice not expressing *Cre* (fl/fl mice) were used as controls.

Animals were kept in a temperature-controlled 12 h light/dark cycle room with *ad libitum* access to food and water. For BCLA time course and dose-response experiments, fed WT mice were treated with BCLA dissolved in 1x phosphate-buffered saline (PBS) at the indicated doses or PBS vehicle alone and liver tissue was collected at the indicated time points. For APAP experiments, mice (n = 4–6 per group) were fasted overnight, then treated i.p. with 300 mg APAP per kg body mass (mg/kg) dissolved in 1x phosphate-buffered saline (PBS). Some WT mice were pre-treated i.p. with the specified doses of BCLA or PBS vehicle for the indicated times before APAP. Blood and liver tissue were collected at 6 h post-APAP. Some liver tissue pieces were fixed in 10% phosphate-buffered formalin for histology, while others were flash-frozen in liquid nitrogen and stored at −80°C for later biochemical analyses. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and Washington University in St. Louis and are consistent with best practices in the *Guide for the Care and Use of Laboratory Animals*.

Biochemistry. Alanine aminotransferase (ALT) was measured in serum using a kit from Pointe Scientific, Inc. (Canton, MI, USA), according to the manufacturer's instructions. Lactate dehydrogenase (LDH) was measured in serum as previously described[31]. For tissue ALT activity, frozen liver tissue was homogenized in 25 mM HEPES buffer with 5 mM EDTA (pH 7.4), 0.1% CHAPS, and protease inhibitors (luepeptin, pepstatin A, and aprotinin) using a bead homogenizer (Thermo Fisher Scientific, Waltham, MA). After centrifugation at 10,000 x *g* and 4ºC for 5 min, ALT activity was measured in the supernatant using the kit from Pointe Scientific,

Inc., mentioned above. Protein concentration in the lysates was measured using the bicinchoninic acid (BCA) assay and the values were used to normalize hepatic ALT activity.

Histology. Formalin-fixed tissue was embedded in paraffin wax and 5 micron sections were mounted on glass slides for hematoxylin and eosin (H&E) staining and immunohistochemistry. H&E staining was performed using a standard protocol.

Western blotting. Western blotting was performed as we previously described (Clemens et al., 2022). Briefly, liver tissue was homogenized in 25 mmol/L HEPES buffer with 5 mmol/L EDTA, 0.1% CHAPS, and a mixture of the protease inhibitors leupeptin, pepstatin A, and aprotinin (pH 7.4; Sigma). Protein concentration was then measured using the BCA assay. After dilution in homogenization buffer, the samples were mixed with reduced Laemmli buffer (Bioworld, Dublin, OH, USA) and boiled for 1 min. Equal amounts of protein (50 μg) were added to each lane of a 4%–20% Tris-glycine gradient gel. After electrophoresis separation, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T). The primary antibodies phospho-JNK (Cat. No. 4668) and JNK (Cat. No. 9252) were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at 1:1,000 dilution in TBS-T with 5% milk. The Cyp2e1 antibody was purchased from Proteintech (Rosemont, IL, USA) (Cat. No. 19937-1-AP). Secondary antibodies were purchased from Li-Cor Biotechnology (Lincoln, NE, USA). Total protein was stained using the Revert Total Protein Stain kit from Li-Cor. All blots were imaged using a Li-Cor Odyssey instrument.

Plasma metabolomics for amino acids and pyruvate. Amino acids and pyruvate were extracted from 20 μL of plasma with 200 μL of methanol containing Try-d8 (1600ng), Phe-d8 (160ng), Tyr-d4 (160ng), Leu-d3 (320ng), Ile-13C6,15N (160ng), Met-d3 (160ng), Val-d8

(400ng), Glu-d3 (2400ng), Thr-13C4 (320ng), Ser-d3 (400ng), Asp-d3 (3200ng), Ala-d4 (1600ng), Asn d3,15N2(3200ng), Gly-d2 (16000ng), Gln-13C5 (3200ng), Pro-d7 (800ng), Cit-d4 (80ng), His 13C6 (400ng), Arg-13C6 (1600ng), Orn-13C5 (400ng), Lys-d8 (400ng), and pyruvic acid-13C5 (80 ng) as the internal standards for Try, Phe, Tyr, Leu, Ile, Met, Val, Glu, Thr, Ser, Asp, Ala, Asn, Gly, Gln, Pro, Cit, His, Arg, Orn, Lys, and pyruvic acid, respectively. The sample aliquots for pyruvic acid were derivatized with o-phenylenediamine to improve mass spectrometric sensitivity. Quality control (QC) samples were prepared by pooling aliquots of study samples and injected every four study samples to monitor instrument performances throughout these analyses.

The analysis of amino acids and pyruvic acid was performed on a Shimadzu 20AD HPLC system and a SIL-20AC autosampler coupled to 4000Qtrap mass spectrometer (AB Sciex) operated in positive multiple reaction monitoring (MRM) mode. Data processing was conducted with Analyst 1.6.3 (Applied Biosystems). The relative quantification data for all analytes were presented as the peak ratio of each analyte to the internal standard. The Metabolomics Facility of Washington University School of Medicine performed the plasma metabolomic analysis.

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

RESULTS

ALT2 deficiency alone has no effect on APAP hepatotoxicity. To test the role of ALT2 in APAP-induced liver injury, we first developed liver-specific ALT2 KO mice and loss of ALT2 protein was confirmed by western blotting (Fig. 2A). These mice were viable and outwardly normal, as previously reported[29]. We then treated the ALT2 KO mice and wildtype littermates with APAP and measured liver injury 6 h later. This early time point was chosen because mitochondrial dysfunction is known to drive early APAP hepatotoxicity. To our surprise, there was no difference in injury between the ALT2 KO animals and their wild type littermates, as indicated by serum ALT, serum LDH, and histology (Fig. 2B-E), and ALT2 KO did not affect APAP bioactivation, as indicated by APAP-protein adducts (mean±SE: 1.7±0.08 vs. 1.6±0.05 nmol/mg protein for WT vs. ALT2 KO, respectively). Although it may seem surprising that ALT2 KO did not itself reduce total serum ALT activity, that is consistent with the fact that ALT1 is the dominant isoform released into serum during liver injury[32-34], accounting for >90% of total serum ALT[34]. Importantly, ALT1 release was unaffected in the KO mice (Fig. 2A). Altogether, our data indicate that ALT2 deletion alone has no effect on the severity of early APAP hepatotoxicity.

β-*chloro*-l-*alanine dose-dependently reduces ALT activity in the liver.* It is possible that conversion of alanine to pyruvate by ALT1 in the cytosol compensates for loss of ALT2. Thus, we next wanted to test the effect of total ALT inhibition on APAP hepatotoxicity. BCLA is an alanine analog originally developed to study amino acid metabolism and enzyme catalysis and irreversibly inhibits ALT activity[35]. Although it is commonly used *in vitro*, little data are available describing its effects *in vivo*, much less within the liver. Therefore, to determine if

BCLA is an effective inhibitor of hepatic ALT activity in mice, we performed time course and dose-response studies (Fig. 3). First, we treated mice with 20 mg/kg BCLA and measured total ALT activity in the liver 4 h and 24 h later. While BCLA had no significant effect at 4 h, it did reduce enzyme activity compared to vehicle treatment at 24 h (Fig. 3A). Based on this, we chose to use 24 h pre-treatment moving forward. Next, we treated mice with increasing doses of BCLA (0, 20, 40, and 100 mg/kg) or with vehicle control and again measured hepatic ALT activity 24 h later. We also treated some mice with two doses of 100 mg/kg BCLA; the first dose 48 h before ALT measurement and the second 24 h before (100x2). BCLA dose-dependently decreased hepatic ALT activity (Fig. 3B), achieving maximum effect at the 100 mg/kg dose (either as a single or double pre-treatment). Based on these data, we chose 24 h and 48 h pre-treatments with 100 mg/kg BCLA as our treatment paradigms to test the effect of ALT activity in APAP toxicity.

β-*chloro*-l-*alanine reduces serum and liver ALT activity but does not protect after APAP overdose.* To determine if combined ALT1 and ALT2 activity has a role in acute liver injury, we pre-treated mice with 100 mg/kg BCLA for either 24 or 48 h (as described above), administered 300 mg/kg APAP to induce hepatotoxicity, and again measured liver injury 6 h post-APAP. Although the single, 24 h pre-treatment with BCLA reduced both serum and hepatic ALT activity >50%, it had no effect on serum LDH (Fig. 4A,B) nor on necrosis visualized and quantified by histology (Fig. 4C,D). Similar data were observed for the 48 h, two-dose pre-treatment (Fig. 4E,F). In addition, BCLA pre-treatment had no effect on APAP bioactivation, as indicated by APAP-protein adducts in the liver (mean±SE: 0.98±0.06 vs. 0.98±0.05 nmol/mg protein for APAP+Veh vs. APAP+BCLA, respectively). Together, these data indicate that

pharmacologic inhibition of total hepatic ALT activity has little or no effect on APAP-induced acute liver injury in mice.

Combined ALT2 and MPC complex deficiency worsens APAP hepatotoxicity. As described in the Introduction, pyruvate primarily enters the mitochondrial matrix via the MPC, a heterodimer in the inner mitochondrial membrane that is made up of the subunits MPC1 and MPC2[25,36,37]. To determine if the MPC has a role in APAP toxicity, we next generated MPC2 KO mice. Because the MPC subunits are mutually required for each other's stability, deletion of MPC2 essentially leads to double knockout of MPC1 and MPC2[36]. Therefore, we refer to these mice as simply MPC KO animals. We have previously shown that loss of the MPC in the liver is well tolerated and that compensation by ALT2-mediated pyruvate-alanine cycling allows these mice to circumvent the loss of the MPC in hepatocytes[30]. Therefore, we generated another, novel line of liver-specific combined ALT2-MPC KO (CKO) mice to further test for redundancy between these sources of mitochondrial pyruvate. Importantly, these mice actually have a triple KO phenotype, since they are deficient for ALT2, MPC2, and indirectly for MPC1[30].

We then treated the WT littermates, MPC KO, and CKO mice with 300 mg/kg APAP and measured injury as before. While MPC KO alone did not alter APAP-induced liver injury compared to WT littermate controls, APAP toxicity was severely exacerbated in the CKO mice based on serum ALT and LDH activity (Fig. 5A,B) and histology (Fig. 5C,D). Importantly, this effect was not due to increased APAP bioactivation to NAPQI as there were was no significant difference in total glutathione between genotypes (Fig. 6A) and APAP-protein adducts were actually lower in the MPC KO and CKO animals (Fig. 6B). Notably, there were no differences in the levels of CYP2E1 – which is required for NAPQI formation – either (Fig. 5C,D), indicating

that the reduced adduct concentrations in the knockout mice were likely due to reduced P450 activity rather than decreased expression. However, despite the reduced protein alkylation, the proportion of glutathione in the oxidized form was ≥3-fold greater in the CKO mice (Fig. 6E) and the amount of phosphorylated JNK was the same (Fig. 6F,G), indicating that a similar or greater degree of oxidative stress developed in the CKO animals.

To assess the effects of MPC KO and CKO on pyruvate and the plasma metabolome, we measured pyruvate and amino acids in plasma after APAP injury. Plasma was chosen because cytosolic fractions would be the ideal specimen but it would be difficult to isolate those fractions from the liver without some contamination from mitochondrial small molecules like pyruvate, especially in the fragile APAP-injured liver, but it is reasonable to expect that some would be exported from the cytosol to blood. Plasma pyruvate was dramatically increased by APAP treatment in WT mice, which is consistent with mitochondrial damage and impaired metabolism, but this was not exacerbated by MPC KO (Fig. 7). In contrast, pyruvate concentrations were further increased 1.9-fold in CKO animals compared to WT controls after APAP treatment (Fig. 7), which is consistent with prior reports that ALT activity is functionally compensating for loss of the MPC to support pyruvate metabolism in liver of mice[30].

Interestingly, at the time point assessed, APAP treatment had no effect on the plasma concentrations of amino acids (Fig. 7). Further, compared to wild-type controls, deletion of the MPC by itself had no significant effect on amino acids in plasma after APAP. In contrast, the abundance of several amino acids was affected in the CKO mice. Consistent with the loss of ALT2 and pyruvate-alanine cycling, combined loss of the MPC and ALT2 in CKO mice resulted in a marked increase in plasma alanine compared to WT and MPC KO (Fig. 7). Proline, tyrosine, and lysine were also increased, while tryptophan was decreased, in the CKO mice compared to

WT and MPC KO. Finally, CKO mice exhibited increased abundance of several amino acids that are intermediates in the urea cycle (aspartate, ornithine, and citrulline), potentially suggesting increased flux through this pathway to dispose of amino acids. None of these effects except the increases in urea cycle intermediates were observed in the absence of APAP treatment (Fig. 8A). These metabolomic data are consistent with a significant disruption of pyruvate metabolism in the CKO mice after APAP overdose by inhibition of pyruvate-alanine cycling.

Combined ALT2/MPC deficiency decreases total hepatic glutathione synthesis.

Reduction of mitochondrial pyruvate content is a likely explanation for the increased injury in CKO mice. However, our observation that the injury was significantly worse in those animals despite even having much lower protein binding led us to consider that other mechanisms in addition to or resulting from pyruvate disruption may also be involved. Glutamate is one of the three amino acids that make up glutathione. Importantly, glutamate is synthesized in the mitochondrial matrix by ALT2 activity during the conversion of alanine to pyruvate. Prior work has also demonstrated that inhibition of the MPC complex enhances use of glutamine/glutamate in the TCA cycle to compensate for loss of pyruvate as a substrate[38]. Importantly, enhanced glutaminolysis was demonstrated in MPC1 KO cells and the effect would likely be even more pronounced in combined ALT2/MPC deficiency. Thus, we hypothesized that the greater injury in DKO mice despite reduced APAP-protein adducts is partially due to impaired glutathione synthesis in addition to disrupted pyruvate metabolism. To test that, we measured basal glutathione in liver tissue from untreated CKO mice and WT littermates. Consistent with our hypothesis, basal hepatic glutathione content was decreased 22% in the CKO animals (Fig. 8B). These data suggest that glutamine/glutamate is diverted away from glutathione synthesis and

toward the TCA cycle to compensate for loss of mitochondrial pyruvate. The latter would also explain why total pyruvate levels are unchanged in the CKO mice at baseline (Fig. 8A).

DISCUSSION

Since it was first applied clinically by Karmen et al. and De Ritis et al. in 1955[39,40], ALT has primarily been thought of as simply a biomarker of liver injury[28]. The roles of ALT enzymes in disease pathophysiology are rarely considered. However, these enzymes are critical for pyruvate-alanine cycling, which is important in maintenance of intermediary mitochondrial metabolism. Here, we explored the effects of ALT blockade in APAP toxicity and demonstrated that the ALT enzymes and the MPC likely share a critical role in limiting APAP-induced liver injury.

Increasing evidence indicates that conservation of mitochondrial metabolism is a possible therapeutic strategy to limit APAP hepatotoxicity. First, it has been demonstrated using NMR studies that the current standard-of-care treatment for APAP overdose, *N*-acetyl-*l*-cysteine (NAC), reduces APAP toxicity in part by maintaining mitochondrial energy metabolism by acting as a pyruvate precursor[22,41,42]. Second, treatment with exogenous pyruvic acid or ethyl pyruvate reduces NAPQI cytotoxicity in HepG2 cells[23]. Finally, Duan et al.[24] recently observed that mice with constitutive PDH activation due to PDK4 deficiency are protected against APAP-induced injury. Since alanine is a significant precursor for synthesizing pyruvate, this led us to the hypothesis that ALT-mediated pyruvate synthesis and/or pyruvate-alanine cycling are important endogenous mechanisms to limit APAP toxicity. While genetic deletion of ALT2 and pharmacologic inhibition of both ALT enzymes did not impact the severity of liver injury post-APAP, our studies using CKO mice indicate that the MPC and ALT2 are redundant sources of

mitochondrial pyruvate, such that inhibiting either alone is insufficient to cause an observable effect on toxicity.

To ensure that the increased toxicity in CKO mice was not simply due to increased NAPQI formation and protein binding, we measured total glutathione and APAP-protein adducts in the liver after APAP overdose. To our surprise, far from being increased, adducts were actually decreased 65% in CKO mice compared to WT littermates, despite having greater injury and equivalent CYP2E1 levels. The reason for this is unclear, but again there are at least two possible explanations. First, disrupting mitochondrial pyruvate could result in reduced TCA cycle flux and therefore lower NADPH production. This would reduce CYP2E1 activity because NADPH is required in the P450 catalytic cycle to form NAPQI. Second, it is possible that stress caused by impaired mitochondrial function in CKO mice stimulates autophagy, which is known to remove APAP-protein adducts and thereby protect against APAP toxicity[43]. In either case, it is apparent that the compromising effects of combined ALT2 and MPC deficiency were so severe that they overcame the protective effect of lower adducts and ultimately caused greater injury.

It is likely that, in addition to further disrupting mitochondrial function, ALT2/MPC KO impairs glutathione synthesis. Glutamate is a product of the conversion of alanine to pyruvate by the ALT reaction in the mitochondrial matrix that is absent in CKO mice (Fig. 1). Prior work has also demonstrated that glutamine/glutamate flux into the TCA is increased in hepatocytes lacking the MPC[44], potentially as a compensatory mechanism to maintain TCA cycle activity. Tompkins et al.[38] previously demonstrated that treatment with the MPC inhibitor UK-5099 moderately decreased basal glutathione levels in Hepa1-6 cells, while both UK-5099 and genetic deletion of MPC1 dramatically reduced glutathione re-synthesis in primary hepatocytes after depletion with buthionine sulfoximine. Indeed, we observed that basal hepatic glutathione levels in our CKO

mice were decreased to a similar extent as in the UK-5099-treated cells in the Tompkins study. While the modest basal glutathione depletion may or may not be enough on its own to drive greater injury, impaired re-synthesis of glutathione after APAP-induced depletion would certainly render the CKO animals more vulnerable to oxidative stress at later time points, when glutathione levels would normally recover. This has been observed before in female mice[45] and Fas receptor-deficient mice[46]. Furthermore, it would explain why JNK phosphorylation – which is stimulated by the early oxidative stress and peaks around 2-3 h after APAP overdose[47,48] – was the same between the CKO and littermate controls at 6 h in our study despite greater glutathione oxidation in the CKO animals. Thus, overall, our results indicate that pyruvate is not only important to conserve mitochondrial function during APAP hepatotoxicity, but is also a glutamate-sparing TCA intermediate that supports glutathione levels.

CONCLUSIONS

Here, we have demonstrated that pyruvate-alanine cycling and the MPC complex are critical redundant systems to limit APAP toxicity, likely by conserving mitochondrial metabolism as well as glutathione synthesis. The data reinforce the well-known central role of mitochondria in APAP toxicity and add to our growing understanding of the importance of deranged pyruvate metabolism in the pathophysiology of acute liver injury. Enhancing pyruvate availability or metabolism may represent a promising novel approach to treat APAP hepatotoxicity.

REFERENCES

1. Kaufman DW, Kelly JP, Rosenberg L, Anderson TE, Mitchell AA. Recent patterns of medication use in the ambulatory adult population of the United States: The Slone survey. J Am Med Assoc 2002;287:337–344.

2. Vernacchio L, Kelly JP, Kaufman DW, Mitchell AA. Medication use among children <12 years of age in the United States: Results from the Slone Survey. Pediatrics 2009;124:446–454.

3. Walsh A, Edwards H, Fraser J. Over-the-counter medication use for childhood fever: A cross-sectional study of Australian parents. J Paediatr Child Health 2007;43:601–606.

4. Barakat-Haddad C, Siddiqua A. Prévalence et facteurs prédictifs de l'automédication chez les adolescents aux Émirats arabes unis. East Mediterr Heal J 2017;23:744–753.

5. Mehuys E, Crombez G, Paemeleire K, Adriaens E, Van Hees T, Demarche S, Christiaens T, Van Bortel L, Van Tongelen I, Remon JP, Boussery K. Self-Medication With Over-the-Counter Analgesics: A Survey of Patient Characteristics and Concerns About Pain Medication. J Pain 2019;20:215–223.

6. Stravitz RT, Lee WM. Acute liver failure. *The Lancet* 2019;394:869–881.

7. McGill MR, Hinson JA. The development and hepatotoxicity of acetaminophen: reviewing over a century of progress. Drug Metab Rev . Epub ahead of print 2020. DOI: 10.1080/03602532.2020.1832112.

8. Ramachandran A, Jaeschke H. A mitochondrial journey through acetaminophen hepatotoxicity. Food Chem Toxicol;140 . Epub ahead of print June 1, 2020. DOI: 10.1016/j.fct.2020.111282.

9. Iorga A, Dara L. Cell death in drug-induced liver injury. Adv Pharmacol 2019;85:31–74.

10. McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H. Acetaminophen-induced liver injury in rats and mice: Comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. Toxicol Appl Pharmacol 2012;264:387–394.

11. Xie Y, McGill MR, Du K, Dorko K, Kumer SC, Schmitt TM, Ding W-X, Jaeschke H. Mitochondrial protein adducts formation and mitochondrial dysfunction during N-acetyl-m-aminophenol (AMAP)-induced hepatotoxicity in primary human hepatocytes. Toxicol Appl Pharmacol 2015;289:213–222.

12. Meyers LL, Beierschmitt WP, Khairallah EA, Cohen SD. Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. Toxicol Appl Pharmacol 1988;93:378–87.

13. Jaeschke H. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. J Pharmacol Exp Ther 1990;255:935–41.

14. Nguyen NT, Du K, Akakpo JY, Umbaugh DS, Jaeschke H, Ramachandran A. Mitochondrial protein adduct and superoxide generation are prerequisites for early activation of c-jun N-terminal kinase within the cytosol after an acetaminophen overdose in mice. Toxicol Lett 2021;338:21–31.

15. Gunawan BK, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. c-Jun N-Terminal Kinase Plays a Major Role in Murine Acetaminophen Hepatotoxicity. Gastroenterology 2006;131:165–178.

16. Iorga A, Donovan K, Shojaie L, Johnson H, Kwok J, Suda J, Lee BT, Aghajan M, Shao L, Liu ZX, Dara L. Interaction of RIPK1 and A20 modulates MAPK signaling in murine acetaminophen toxicity. J Biol Chem;296 . Epub ahead of print January 1, 2021. DOI: 10.1016/j.jbc.2021.100300.

17. Win S, Than TA, Min RWM, Aghajan M, Kaplowitz N. c-Jun N-terminal kinase mediates mouse liver injury through a novel Sab (SH3BP5)-dependent pathway leading to inactivation of intramitochondrial Src. Hepatology 2016;63:1987–2003.

18. Kon K, Kim JS, Jaeschke H, Lemasters JJ. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatology 2004;40:1170–1179.

19. Placke ME, Ginsberg GL, Wyand DS, Cohen SD. Ultrastructural Changes during Acute Acetaminophen-Induced Hepatotoxicity in the Mouse: A Time and Dose Study. Toxicol Pathol 1987;15:431–438.

20. Bajt ML, Cover C, Lemasters JJ, Jaeschke H. Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol Sci 2006;94:217–225.

21. Gujral JS, Knight TR, Farhood A, Bajt ML, Jaeschke H. Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? Toxicol Sci 2002;67:322–8.

22. Saito C, Zwingmann C, Jaeschke H. Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine. Hepatology 2010;51:246–254.

23. Nagatome M, Kondo Y, Kadowaki D, Saishyo Y, Irikura M, Irie T, Ishitsuka Y. Ethyl pyruvate attenuates acetaminophen-induced liver injury in mice and prevents cellular injury induced by N-acetyl-p-benzoquinone imine, a toxic metabolite of acetaminophen, in hepatic cell lines. Heliyon;4 . Epub ahead of print February 1, 2018. DOI: 10.1016/j.heliyon.2018.e00521.

24. Duan L, Ramachandran A, Akakpo JY, Woolbright BL, Zhang Y, Jaeschke H. Mice deficient in pyruvate dehydrogenase kinase 4 are protected against acetaminophen-induced hepatotoxicity. Toxicol Appl Pharmacol;387 . Epub ahead of print January 15, 2020. DOI: 10.1016/j.taap.2019.114849.

25. McCommis KS, Finck BN. Mitochondrial pyruvate transport: A historical perspective and future research directions. *Biochemical Journal* 2015;466:443–454.

26. Sharma G, Wu CY, Wynn RM, Gui W, Malloy CR, Sherry AD, Chuang DT, Khemtong C. Real-time hyperpolarized 13 C magnetic resonance detects increased pyruvate oxidation in pyruvate dehydrogenase kinase 2/4-double knockout mouse livers. Sci Rep;9. Epub ahead of print December 1, 2019. DOI: 10.1038/S41598-019-52952-6.

27. Cappel DA, Deja S, Duarte JAG, Kucejova B, Iñigo M, Fletcher JA, Fu X, Berglund ED, Liu T, Elmquist JK, Hammer S, Mishra P, Browning JD, Burgess SC. Pyruvate-Carboxylase-Mediated Anaplerosis Promotes Antioxidant Capacity by Sustaining TCA Cycle and Redox Metabolism in Liver. Cell Metab 2019;29:1291-1305.e8.

28. McGill MR. The past and present of serum aminotransferases and the future of liver injury biomarkers. EXCLI J 2016;15:817–828.

29. Martino MR, Gutiérrez-Aguilar M, Yiew NKH, Lutkewitte AJ, Singer JM, McCommis

KS, Ferguson D, Liss KHH, Yoshino J, Renkemeyer MK, Smith GI, Cho K, Fletcher JA, Klein S, Patti GJ, Burgess SC, Finck BN. Silencing alanine transaminase 2 in diabetic liver attenuates hyperglycemia by reducing gluconeogenesis from amino acids. Cell Rep. 2022;39:110733.

30. McCommis KS, Chen Z, Fu X, McDonald WG, Colca JR, Kletzien RF, Burgess SC, Finck BN. Loss of mitochondrial pyruvate carrier 2 in the liver leads to defects in gluconeogenesis and compensation via pyruvate-alanine cycling. Cell Metab 2015;22:682–694.

31. Vazquez JH, Kennon-McGill S, Byrum SD, Mackintosh SG, Jaeschke H, Williams DK, Lee WM, Dranoff JA, McGill MR. Proteomics Indicates Lactate Dehydrogenase is Prognostic in Acetaminophen-induced Acute Liver Failure Patients and Reveals Altered Signaling Pathways. Toxicol Sci . Epub ahead of print February 16, 2022. DOI: 10.1093/toxsci/kfac015.

32. Miyazaki T, Honda A, Ikegami T, Hara T, Saitoh Y, Hirayama T, Doy M, Matsuzaki Y. Determination of enzymatic source of alanine aminotransferase activity in serum from dogs with liver injury. J Pharmacol Toxicol Methods. 2009;60:307-15.

33. Yang RZ, Park S, Reagan WJ, Goldstein R, Zhong S, Lawton M, Rajamohan F, Qian K, Gong DW. Alanine aminotransferase isoenzymes: molecular cloning and quantitative analysis of tissue expression in rats and serum elevation in liver toxicity. Hepatology. 2009;49:598-607.

34. Rafter I, Gråberg T, Kotronen A, Strömmer L, Mattson CM, Kim RW, Ehrenbord E, Andersson HB, Yki-Järvinen, Schuppe-Koistinen I, Ekblom B, Cotgreave I, Glinghammer

B. Isoform-specific alanine aminotransferase measurement can distinguish hepatic from extrahepatic injury in humans. 2012;30:1241-9.

35. Gregerman RI, Christensen HN. Enzymatic and non-enzymatic dehydrochlorination of β-chloro-l-alanine. J Biol Chem 1956;220:765–774.

36. Herzig S, Raemy E, Montessuit S, Veuthey JL, Zamboni N, Westermann B, Kunji ER, Martinou JC. Identification and functional expression of the mitochondrial pyruvate carrier. 2012;337:93-96.

37. Bricker DK, Taylor EB, Schell JC, Orsak T, Boutron A, Chen YC, Cox JE, Cardon CM, Van Vranken JG, Dephoure N, Redin C, Boudina S, Gygi SP, Brivet M, Thummel CS, Rutter J. A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, Drosophila, and humans. 2012;337:96-100.

38. Tompkins SC, Sheldon RD, Rauckhorst AJ, Noterman MF, Solst SR, Buchanan JL, Mapuskar KA, Pewa AD, Gray LR, Oonthonpan L, Sharma A, Scerbo DA, Dupuy AJ, Spitz DR, Taylor EB. Disrupting Mitochondrial Pyruvate Uptake Directs Glutamine into the TCA Cycle away from Glutathione Synthesis and Impairs Hepatocellular Tumorigenesis. Cell Rep 2019;28:2608-2619.e6.

39. Karmen A, Wroblewski F, LaDue JS. Transaminase activity in human blood. J Clin Invest 1955;34:126–131.

40. De Ritis F, Coltori M, Giusti G. Transaminase activity of human serum in viral hepatitis; preventive note. Minerva Med 1955;46:1207–129.

41. Zwingmann C, Bilodeau M. Metabolic insights into the hepatoprotective role of N-acetylcysteine in mouse liver. Hepatology 2006;43:454–463.

42. McGill MR, Li F, Sharpe MR, Williams CD, Curry SC, Ma X, Jaeschke H. Circulating acylcarnitines as biomarkers of mitochondrial dysfunction after acetaminophen overdose in mice and humans. Arch Toxicol 2014;88:391–401.

43. Ni HM, McGill MR, Chao X, Du K, Williams JA, Xie Y, Jaeschke H, Ding WX. Removal of acetaminophen protein adducts by autophagy protects against acetaminophen-induced liver injury in mice. J Hepatol 2016;65:354–362.

44. Gray LR, Sultana MR, Rauckhorst AJ, Oonthonpan L, Tompkins SC, Sharma A, Fu X, Miao R, Pewa AD, Brown KS, Lane EE, Dohlman A, Zepeda-Orozco D, Xie J, Rutter J, Norris AW, Cox JE, Burgess SC, Potthoff MJ, Taylor EB. Hepatic Mitochondrial Pyruvate Carrier 1 Is Required for Efficient Regulation of Gluconeogenesis and Whole-Body Glucose Homeostasis. Cell Metab. 2015;22:669-81.

45. Du K, Williams CD, McGill MR, Jaeschke H. Lower susceptibility of female mice to acetaminophen hepatotoxicity: Role of mitochondrial glutathione, oxidant stress and c-jun N-terminal kinase. Toxicol Appl Pharmacol 2014;281:58–66.

46. Williams CD, McGill MR, Farhood A, Jaeschke H. Fas receptor-deficient lpr mice are protected against acetaminophen hepatotoxicity due to higher glutathione synthesis and enhanced detoxification of oxidant stress. Food Chem Toxicol 2013;58:228–235.

47. Nakagawa H, Maeda S, Hikiba Y, Ohmae T, Shibata W, Yanai A, Sakamoto K, Ogura K, Noguchi T, Karin M, Ichijo H, Omata M. Deletion of Apoptosis Signal-Regulating Kinase 1 Attenuates Acetaminophen-Induced Liver Injury by Inhibiting c-Jun N-Terminal Kinase Activation. Gastroenterology 2008;135:1311–1321.

48. Xie Y, Ramachandran A, Breckenridge DG, Liles JT, Lebofsky M, Farhood A, Jaeschke

H. Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury. Toxicol Appl Pharmacol 2015;286:1–9.

FIGURES

Figure 1. Schematic of mitochondrial pyruvate metabolism. Pyruvate (Pyr) is produced in the cytosol from glycolysis (via phosphoenolpyruvate [PEP]), lactate, and alanine (Ala). It is then transported into mitochondria through the mitochondrial pyruvate carrier (MPC) complex. Pyr can also be generated directly within mitochondria from Ala. Once inside, Pyr is converted to acetyl-CoA, which feeds into the tricarboxylic acid (TCA) cycle, generating NADH, NADPH, and $FADH_2$. Other amino acids, such as glutamine (Gln) and glutamate (Glu), can also enter the TCA cycle via α-ketoglutarate (a-KG) or other intermediaries. Other important reactions not shown: Conversion of TCA cycle intermediate oxaloacetate to PEP which can then return to Pyr or be used for gluconeogenesis. Other abbreviations: Ala, alanine; ALT, alanine aminotransferase; MPC, mitochondrial pyruvate carrier; PC, pyruvate carboxylase; PHD, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; Pyr, pyruvate; TCA, tricarboxylic acid cycle.

Figure 2. ALT2 deficiency does not affect APAP hepatotoxicity. ALT2 KO and wild type (WT) littermate controls were treated with 300 mg/kg APAP to induce liver injury. Hepatic ALT1 and ALT2 levels and serum ALT1 were determined by immunoblotting, and serum ALT and LDH activity were measured at 6 h post-APAP by kinetics. (A) ALT1 and 2 immunoblots. (B) Serum ALT activity. (C) Serum LDH activity. (D) H&E-stained liver sections. (E) Percent necrosis from histology. Data are expressed as mean±SE for n = 4 mice per bar. APAP increased liver injury. *$p < 0.05$ for APAP vs. Veh. NS = Not significant.

Figure 3. BCLA reduces total hepatic ALT activity. Mice were treated with the specified doses of BCLA for the indicated times. Total hepatic ALT activity was measured in liver lysates. (A) Hepatic ALT activity at 4 and 24 h after treatment with 20 mg/kg BCLA. (B) Hepatic ALT

activity at 24 h after treatment with the indicated doses of BCLA. Data are expressed as mean±SE for n = 3-6 mice per bar. *p<0.05 vs. Veh or 0 dose.

Figure 4. BCLA does not affect APAP hepatotoxicity. Mice were pre-treated for 24 h with a single dose of 100 mg/kg BCLA (panels A-D) or for 48 h with two doses of 100 mg/kg BCLA spread 24 h apart (panels E-F), followed by 300 mg/kg APAP to induce liver injury. Serum ALT and LDH and tissue ALT and necrosis were measured at 6 h post-APAP. (A) Serum enzymes. (B) Hepatic ALT activity. (C) H&E-stained liver sections. (D) Percent necrosis from histology. (E) Serum enzymes from 48 h pre-treatment experiment. (F) Hepatic ALT activity from 48 h pre-treatment experiment. Data are expressed as mean±SE for n = 5-6 mice per bar. *p<0.05 vs. Veh or APAP+Veh.

Figure 5. The ALT and MPC systems are critical and redundant to limit APAP hepatotoxicity. MPC2 KO mice, combined ALT2-MPC KO mice (CKO), and wild type (WT) littermates were treated with 300 mg/kg APAP to induce liver injury. ALT and LDH were measured in serum and necrosis was quantified in tissue. (A) Serum ALT. (B) Serum LDH. (C) H&E-stained liver sections. (D) Percent necrosis from histology. Data are expressed as mean±SE for n = 4 mice per bar. *p < 0.05 for the indicated comparisons.

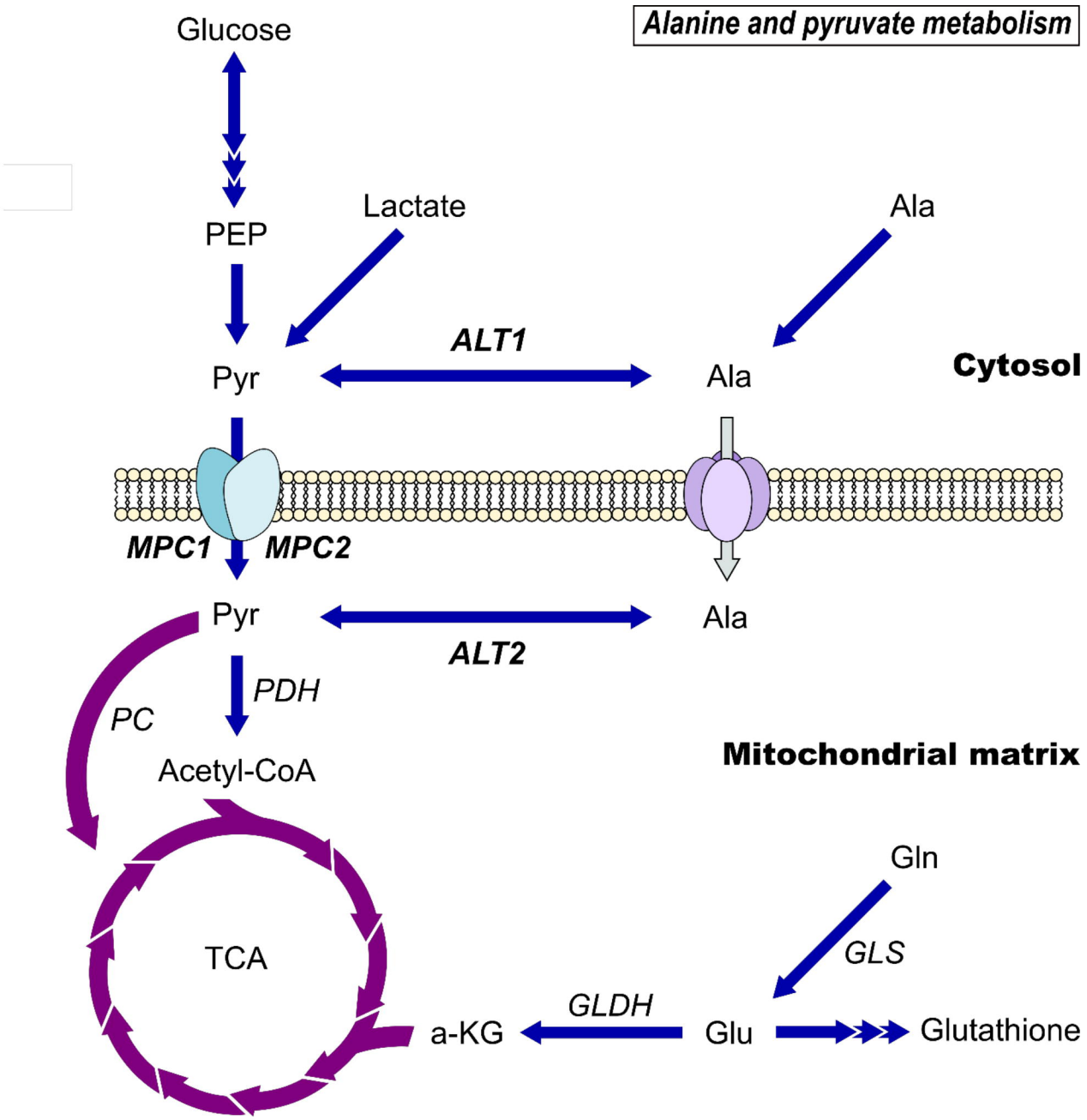
Figure 6. The increased injury in CKO mice is not due to increased APAP bioactivation. MPC2 KO ($MPC2^{-/-}$) mice, combined ALT2-MPC KO mice (CKO), and wild type (WT) littermates were treated with 300 mg/kg APAP to induce liver injury. Some WT mice were treated with vehicle only (Veh). Total hepatic glutathione (GSH+GSSG) and oxidized glutathione (GSSG), APAP-protein adducts (APAP-protein), and JNK phosphorylation were measured at 6 h post-APAP. (A) Total liver glutathione. (B) APAP-protein adducts. (C) CYP2E1 immunoblot and total protein stain. (D) Densitometry for CYP2E1. (E) Percentage of glutathione

in the oxidized form (F) Immunoblots for phosphorylated and total JNK. (G) Densitometry for JNK blots. Data are expressed as mean±SE for n = 4 mice per bar. *$p < 0.05$ vs. Veh. $^{\#}p < 0.05$ vs. WT APAP. $^{\wedge}p < 0.05$ vs. $MPC^{-/-}$ mice.

Figure 7. Pyruvate metabolism is disrupted in APAP toxicity and worsened with ALT2-MPC CKO. MPC2 KO ($MPC2^{-/-}$) mice, combined ALT2-MPC KO mice (CKO), and wild type (WT) littermates were treated with 300 mg/kg APAP to induce liver injury. Some WT mice were treated with vehicle-only (Veh). Serum metabolomics was done by mass spectrometry. Data are expressed as mean±SE for n = 4 mice per bar. *$p<0.05$ vs. WT Veh. $^{\#}p<0.05$ vs. MPC KO. ^$p<0.05$ vs. all other groups.

Figure 8. Combined ALT2-MPC2 deficiency decreases basal glutathione in the liver. MPC2 KO ($MPC2^{-/-}$) mice and wild type (WT) littermates were fasted for 6 h to stabilize metabolite levels and serum metabolomics was done by mass spectrometry. (A) Results for amino acids and pyruvate. (B) Results for total hepatic glutathione. Data are expressed as mean±SE for n = 6 mice per bar. *$p<0.05$ vs. WT.

Fig. 1.


Alanine and pyruvate metabolism
Glucose
PEP
Lactate
Ala
ALT1
Pyr
Ala
Cytosol
MPC1
MPC2
Pyr
Ala
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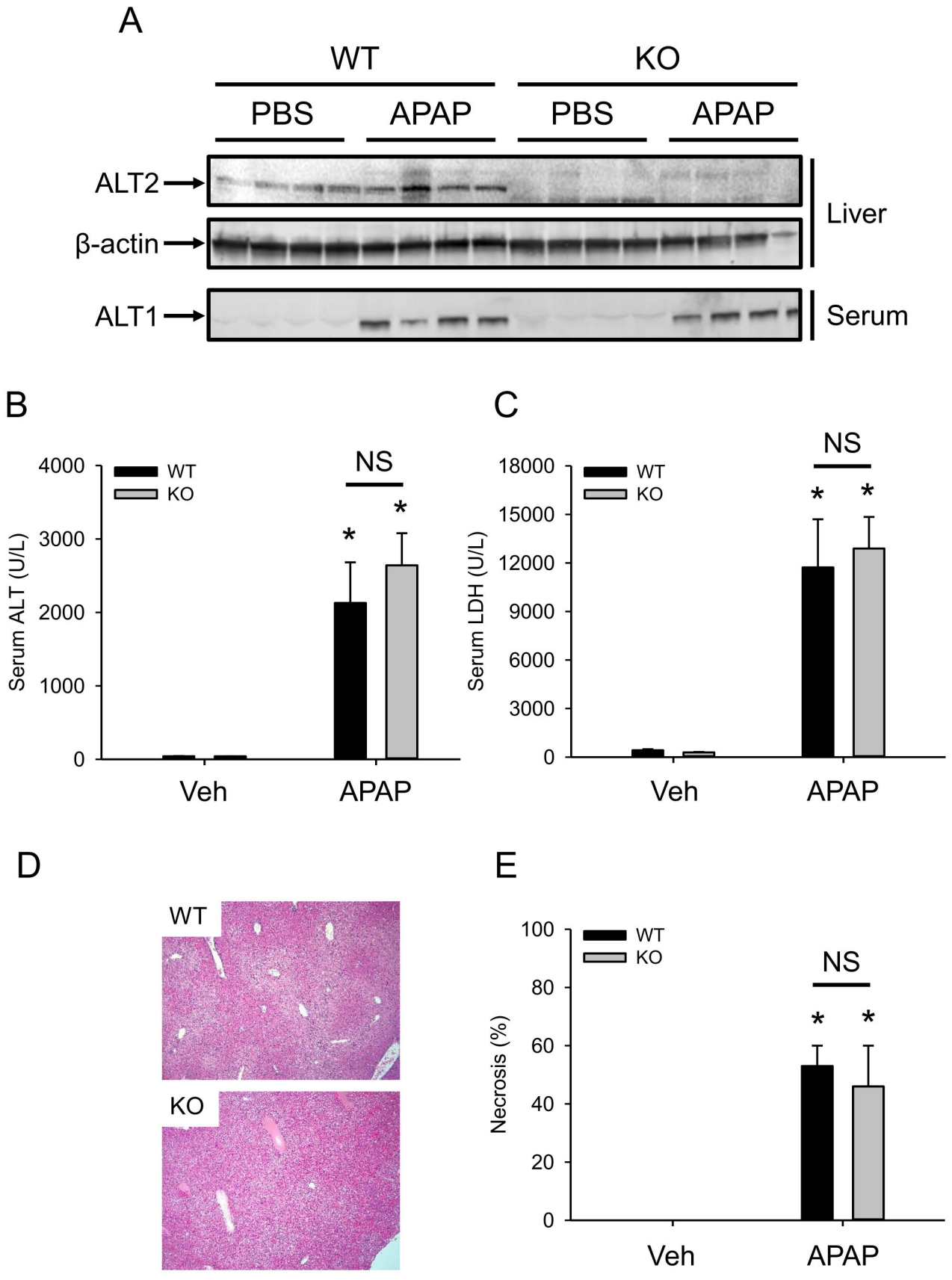
TCA
GLDH
a-KG
Glu
Glutathione

Fig. 2.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WT
KO
PBS
APAP
PBS
APAP
ALT2
β-actin
ALT1
Liver
Serum
B
Serum ALT (U/L)
4000
3000
2000
1000
0
WT
KO
NS
*
*
Veh
APAP
C
Serum LDH (U/L)
18000
15000
12000
9000
6000
3000
0
WT
KO
NS
*
*
Veh
APAP
D
WT
KO
E
Necrosis (%)
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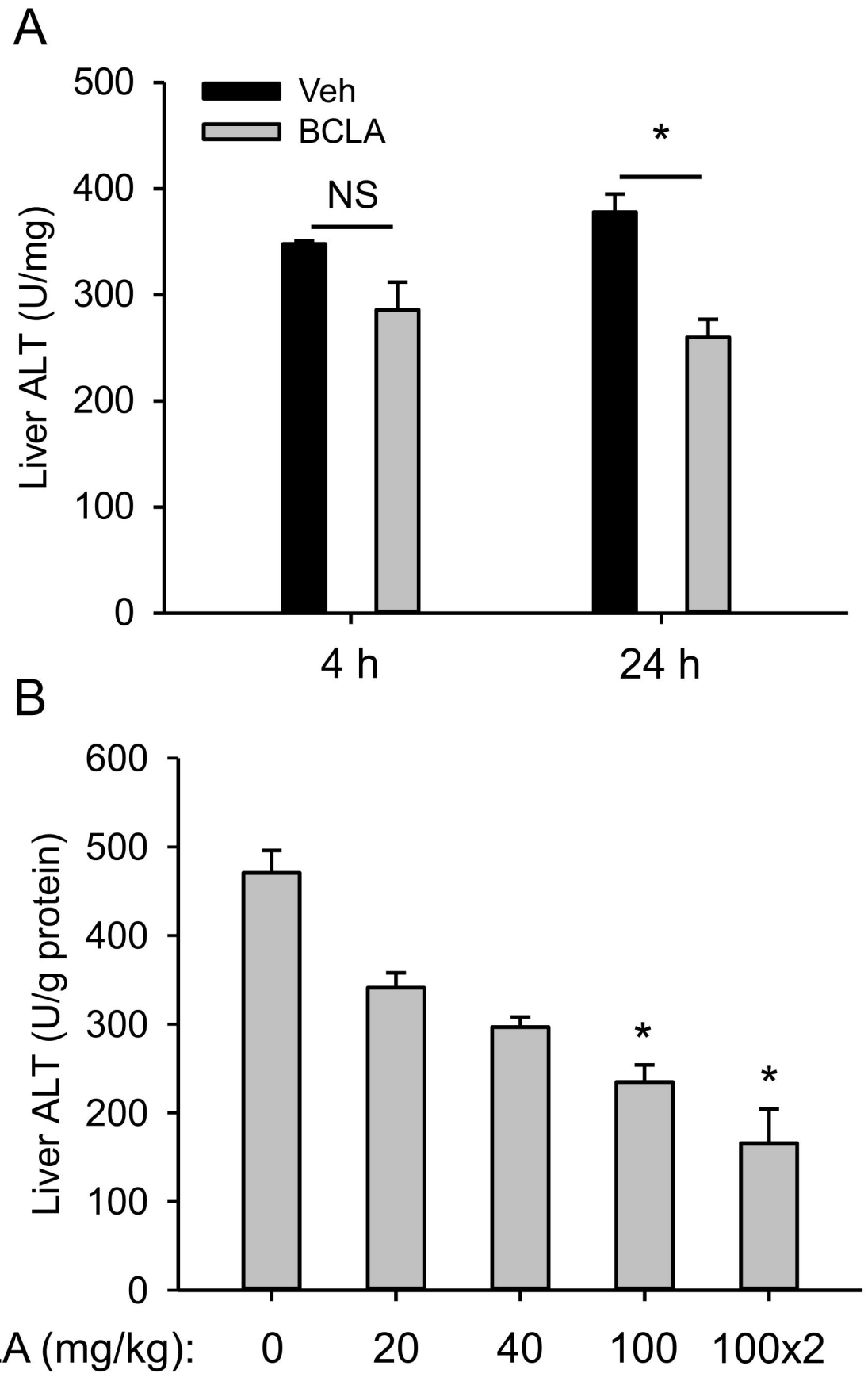
80
60
40
20
0
WT
KO
NS
*
*
Veh
APAP

Fig. 3.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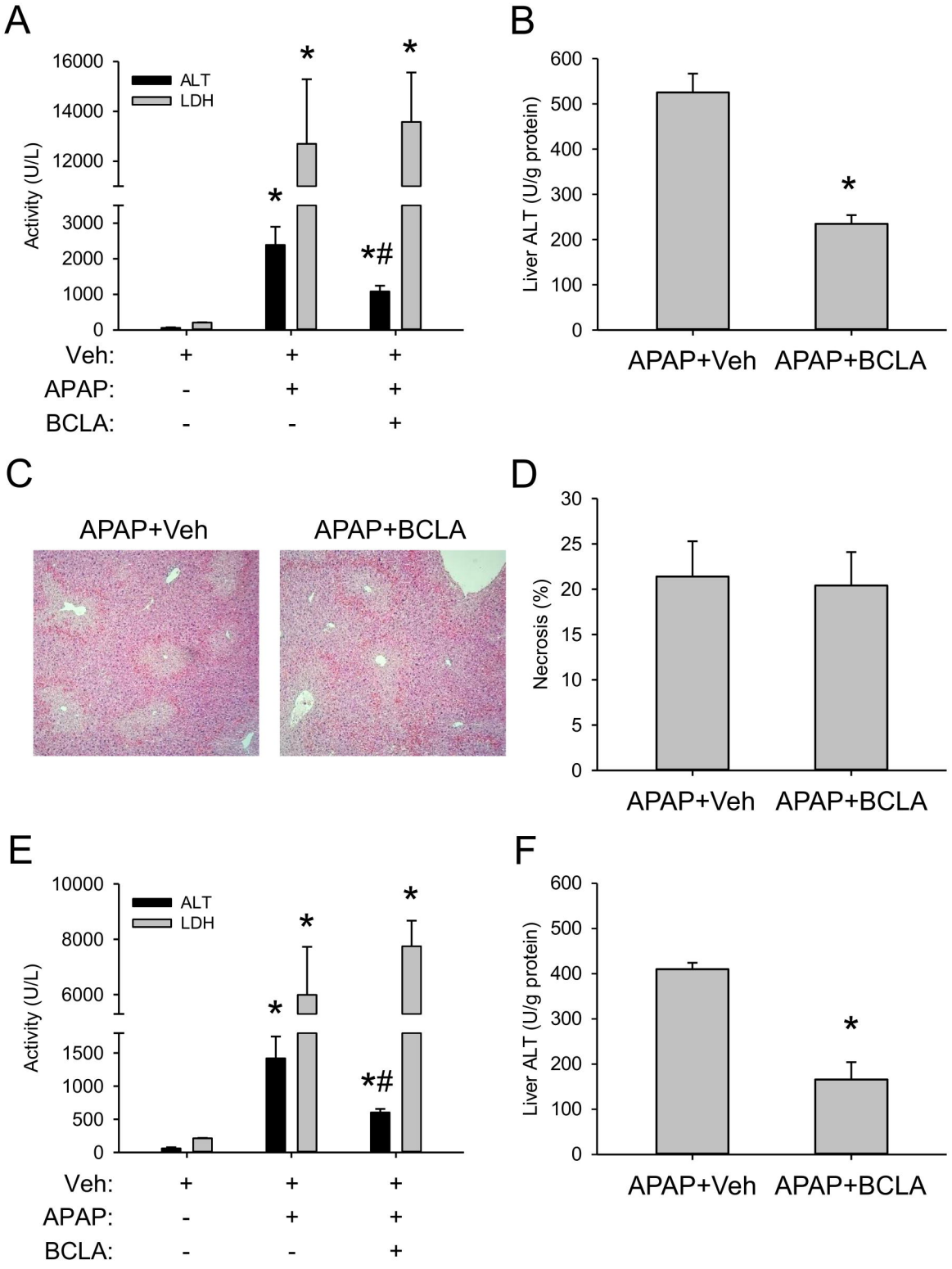
400
300
200
100
0
Liver ALT (U/mg)
Veh
BCLA
NS
*
4 h
24 h
B
600
500
400
300
200
100
0
Liver ALT (U/g protein)
*
*
BCLA (mg/kg):
0
20
40
100
100x2

Fig. 4.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16000
14000
12000
3000
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ALT
LDH
Activity (U/L)
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*#
Veh: + + +
APAP: - + +
BCLA: - - +
B
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500
400
300
200
100
0
Liver ALT (U/g protein)
*
APAP+Veh
APAP+BCLA
C
APAP+Veh
APAP+BCLA
D
30
25
20
15
10
5
0
Necrosis (%)
APAP+Veh
APAP+BCLA
E
10000
8000
6000
1500
1000
500
0
ALT
LDH
Activity (U/L)
*
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Veh: + + +
APAP: - + +
BCLA: - - +
F
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100
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Liver ALT (U/g protein)
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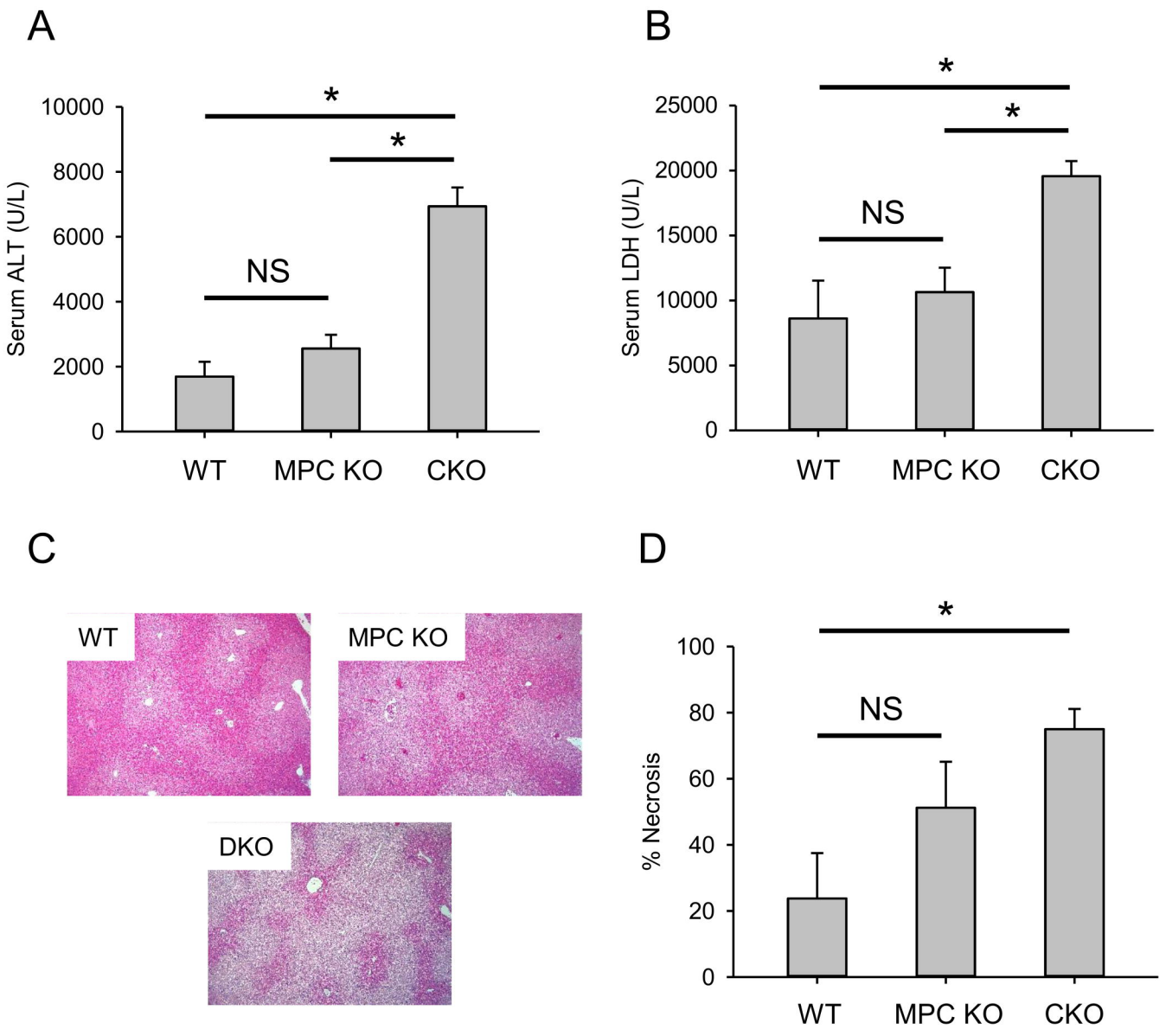
APAP+Veh
APAP+BCLA

Fig. 5.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Serum ALT (U/L)
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6000
4000
2000
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*
*
NS
WT
MPC KO
CKO
B
Serum LDH (U/L)
25000
20000
15000
10000
5000
0
*
*
NS
WT
MPC KO
CKO
C
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% Necrosis
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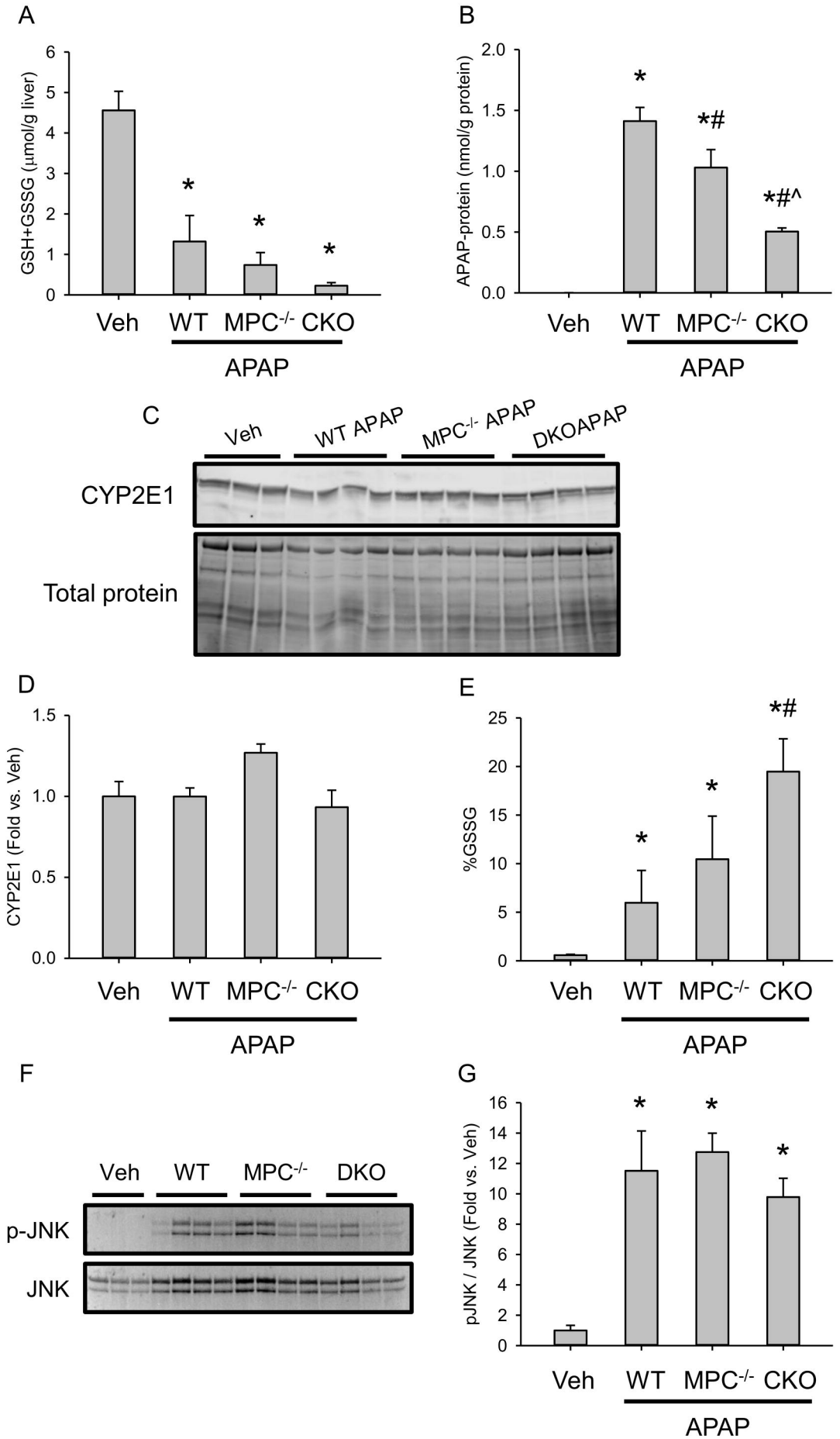
MPC KO
CKO

Fig. 6.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GSH+GSSG (μmol/g liver)
Veh
WT
MPC-/-
CKO
APAP
B
APAP-protein (nmol/g protein)
*#
*#^
C
Veh
WT APAP
MPC-/- APAP
DKOAPAP
CYP2E1
Total protein
D
CYP2E1 (Fold vs. Veh)
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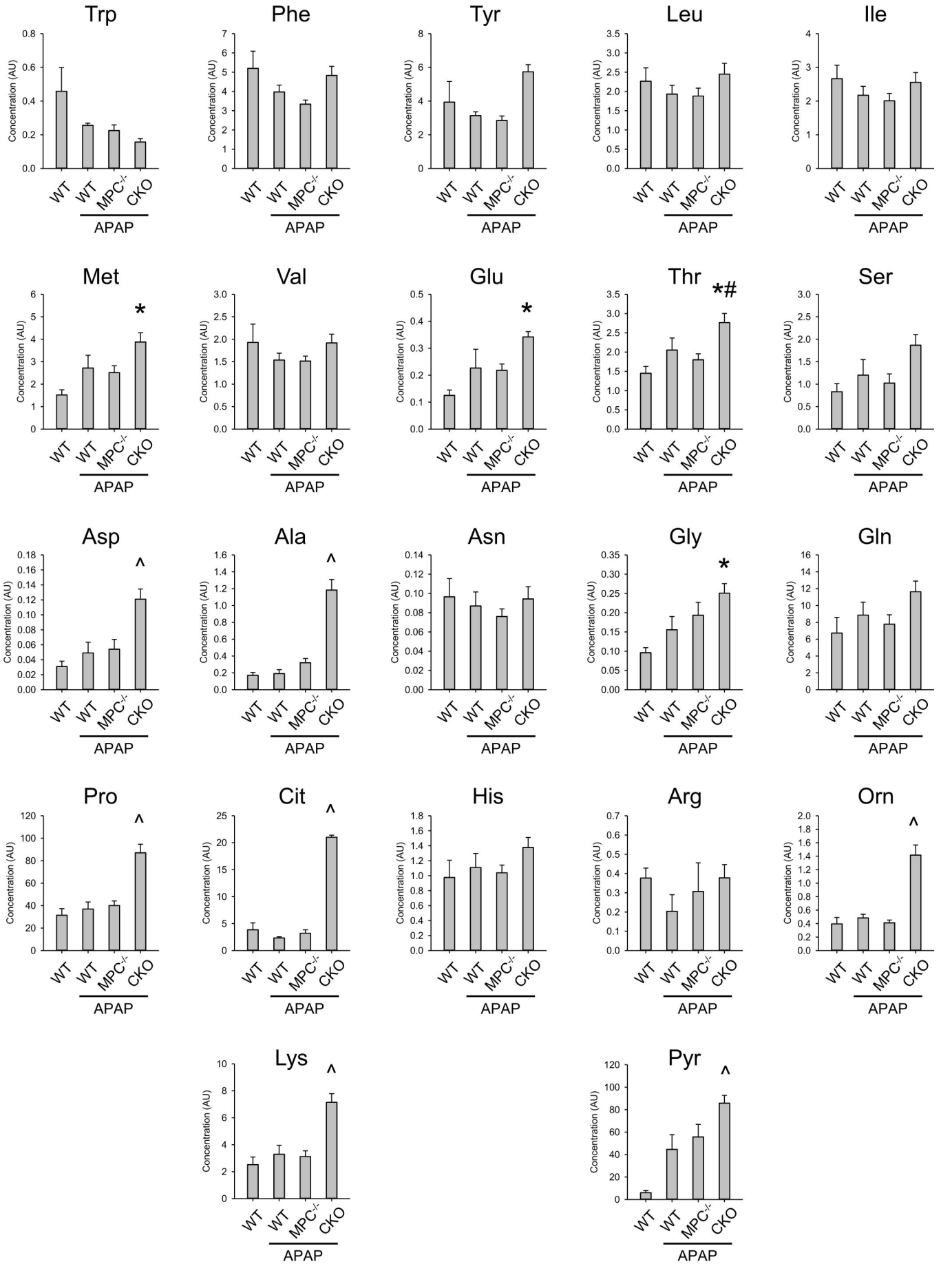
%GSSG
F
p-JNK
JNK
DKO
G
pJNK / JNK (Fold vs. Veh)

Fig. 7.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WT
MPC-/-
CKO
APAP
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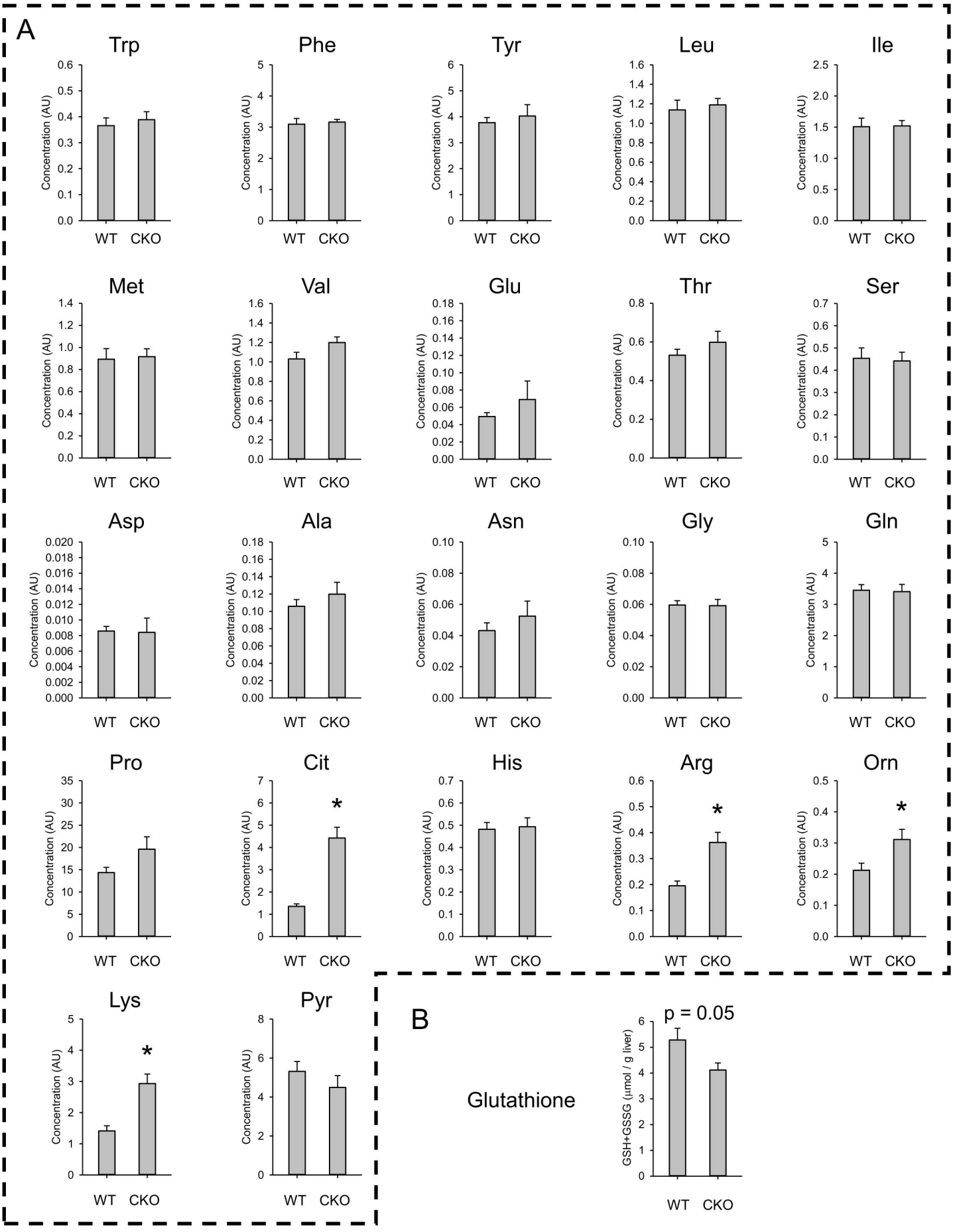
Fig. 8.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Lys
Pyr
Concentration (AU)
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CKO
*
B
Glutathione
p = 0.05
GSH+GSSG (µmol / g liver)