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RESEARCH PAPER

Loss of 5-lipoxygenase activity protects mice against paracetamol-induced liver toxicity

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BACKGROUND AND PURPOSE

Paracetamol (acetaminophen) is the most widely used over-the-counter analgesic and overdosing with paracetamol is the leading cause of hospital admission for acute liver failure. 5-Lipoxygenase (5-LO) catalyses arachidonic acid to form LTs, which lead to inflammation and oxidative stress. In this study, we examined whether deletion or pharmacological inhibition of 5-LO could protect mice against paracetamol-induced hepatic toxicity.

EXPERIMENTAL APPROACH

Both genetic deletion and pharmacological inhibition of 5-LO in C57BL/6J mice were used to study the role of this enzyme in paracetamol induced liver toxicity. Serum and tissue biochemistry, H&E staining, and real-time PCR were used to assess liver toxicity.

KEY RESULTS

Deletion or pharmacological inhibition of 5-LO in mice markedly ameliorated paracetamol-induced hepatic injury, as shown by decreased serum alanine transaminase and aspartate aminotransferase levels and hepatic centrilobular necrosis. The hepato-protective effect of 5-LO inhibition was associated with induction of the antitoxic phase II conjugating enzyme, sulfotransferase2a1, suppression of the pro-toxic phase I CYP3A11 and reduction of the hepatic transporter MRP3. In 5-LO$^{-/-}$ mice, levels of GSH were increased, and oxidative stress decreased. In addition, PPAR α, a nuclear receptor that confers resistance to paracetamol toxicity, was activated in 5-LO$^{-/-}$ mice.

CONCLUSIONS AND IMPLICATIONS

The activity of 5-LO may play a critical role in paracetamol-induced hepatic toxicity by regulating paracetamol metabolism and oxidative stress.

Abbreviations

5-LO, 5-lipoxygenase; Gst, glutathione S-transferase; MRP3, multidrug resistance-associated protein 3; NAPQI, N-acetyl-*p*-benzoquinone-imine; ROS, reactive oxygen species; Sult, sulfotransferase

Tables of Links

TARGETS	
Enzymes[a]	**Transporters**[b]
5-LO, 5-lipoxygenase	MRP3 (ABCC3)
CYP2E1	**Nuclear hormone receptors**[c]
CYP3A4	PPARα
ERK1/2	PPARγ
JNK1/2	

LIGANDS
Paracetamol
Zileuton

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a b c]Alexander *et al.*, 2013a,b,c).

Introduction

Paracetamol (acetaminophen) is the most widely used over-the-counter analgesic. Overdose of paracetamol is the leading cause of hospital admission for acute liver failure (James *et al.*, 2003; Saini *et al.*, 2011). The majority of ingested paracetamol is rapidly metabolized by the conjugating enzymes UDP-glucuronosyltransferase and sulfotransferase (Sult) in the liver to non-toxic compounds, followed by renal and biliary excretion (James *et al.*, 2003; Saini *et al.*, 2011). Less than 10% of paracetamol is bioactivated by phase I cytochrome P450 (CYP) enzymes to the highly reactive intermediate metabolite, N-acetyl-*p*-benzoquinone-imine (NAPQI). NAPQI is detoxified by GSH to form paracetamol cysteine and mercapturate conjugates (Albano *et al.*, 1985). With paracetamol overdose, GSH is depleted, and as a result, NAPQI accumulates and binds to proteins and causes cellular damage (James *et al.*, 2003).

The cytochrome P450 enzymes, CYP1A2, CYP2E1 and CYP3A4, constitute the major pathways converting paracetamol to its reactive metabolite (Patten *et al.*, 1993). Induction of the gene, Cyp1a2, increased paracetamol hepatotoxicity in rodents (Kalhorn *et al.*, 1990). CYP2E1 and CYP3A are the most active P450 forms that convert paracetamol to NAPQI and paracetamol hepatotoxicity was lower in Cyp2e1−/− mice than in wild-type (WT) mice (Lee *et al.*, 1996). In contrast, alcohol, which induces Cyp2e1 expression, has been shown to increase paracetamol hepatotoxicity (McClain *et al.*, 1980). CYP3A has a higher affinity for paracetamol than CYP2E1 (Wolf *et al.*, 2005) and treating animals with inducers of CYP3A, such as dexamethasone and phenobarbital, increased paracetamol hepatotoxicity (Cheng *et al.*, 2009). Therefore, increased levels of CYP3A in the liver may play a role in paracetamol toxicity.

In addition to P450 enzymes, several hepatic uptake and export transporters are implicated in paracetamol hepatotoxicity. Hepatotoxic doses of paracetamol reduced the expression of uptake transporters such as the organic anion-transporting polypeptide 1A4 and increased that of export transporters such as multidrug resistance-associated proteins (MRPs) (Aleksunes *et al.*, 2006). Among the MRPs, the MRP3 was thought to be important in basolateral excretion of paracetamol metabolites. However, despite the involvement of MRP3 in the excretion of paracetamol metabolites, $Mrp3^{-/-}$ mice were more resistant to paracetamol hepatotoxicity, which was explained by a faster repletion of hepatic GSH in the $Mrp3^{-/-}$ mice (Manautou *et al.*, 2005).

Oxidative stress has been suggested to be important in the development of paracetamol toxicity. At therapeutic doses of paracetamol, its metabolite NAPQI rapidly reacts with GSH to form an paracetamol–GSH conjugate and GSH disulfide (GSSG) (Albano *et al.*, 1985). At excessive doses of paracetamol, the amount of NAPQI that is formed depletes hepatic GSH and increases the production of superoxide, H_2O_2 and lipid peroxidation (Hanawa *et al.*, 2008; Afroz *et al.*, 2014; Farag *et al.*, 2015), which may further trigger hepatic toxicity (Holtzman, 1995; Gonzalez, 2007).

The enzyme 5-lipoxygenase (5-LO) initiates the synthesis of LTs from arachidonic acid. 5-LO is activated under conditions that promote lipid peroxidation via depletion of GSH (Werz *et al.*, 1998). On activation, 5-LO catalyses the oxygenation of arachidonic acid to form the epoxide intermediate LTA_4. LTA_4 can be hydrolyzed to LTB_4 or conjugated with GSH to yield LTC_4. Then, LTC_4 is converted to LTD_4. LTB_4, LTC_4 and LTD_4 are important mediators of inflammatory and allergic reactions (Brock, 2005). The synthesis of LTs from arachidonic acid by 5-LO changes the intracellular redox balance and promotes reactive oxygen species (ROS) production (Cho *et al.*, 2011). Several reports showed that inhibition of 5-LO decreased cellular ROS production (Los *et al.*, 1995; Lee *et al.*, 1997). Inhibition of the 5-LO pathway can also divert the metabolism of arachidonate to the other pathways and induce production of alternative metabolites, such as 8(S)-HETE and prostanoids (Vanderhoek *et al.*, 1985; Muga *et al.*, 2000; Larsen *et al.*, 2008), which are endogenous ligands of PPARα, a member of the nuclear receptor superfamily. This receptor controls the expression of a battery of genes involved in energy metabolism, oxidative stress and inflammation (Peters *et al.*, 1997; Lefebvre *et al.*, 2006; Shah *et al.*, 2007; Gonzalez and Shah, 2008; Papi *et al.*, 2012). Activation of PPARα protects against paracetamol-induced liver injury and decreases mitochondrial ROS levels by up-regulating the mitochondrial uncoupling protein 2 (Ucp-2) (D'Arcy, 1997; Nakatani *et al.*, 2002; Gonzalez and Shah, 2008; Mailloux *et al.*, 2011; Patterson *et al.*, 2012).

In this study, we investigated whether deletion or pharmacological inhibition of 5-LO in mice could protect against

paracetamol-induced hepatotoxicity. The beneficial effect of 5-LO inhibition was associated with a pattern of metabolic gene regulation that favoured decreased exposure of the host to the paracetamol-derived metabolic toxicants. The beneficial effect was also associated with reduced ROS production and PPARα activation. Our results suggested that 5-LO might be a therapeutic target for treatment of paracetamol-induced liver toxicity.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the Animal Care and Utilization Committee of the Institute and were approved by this Committee. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 88 animals were used in the experiments described here.

Male C57BL/6J mice (WT) and 5-LO$^{-/-}$ mice on a C57BL/6J background (8-weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). DNA was extracted from the tail of each mouse and analyzed by PCR to confirm the genotype. Mice were housed in the animal facility at West China Hospital, Sichuan University, with freely available food and water, unless otherwise stated. Mice were fasted overnight before oral administration of paracetamol (200 mg kg^{-1}; Sigma, St. Louis, MO). When necessary, C57BL/6J mice or 5-LO$^{-/-}$ mice were treated, by gavage, with the 5-LO inhibitor zileuton (100 mg kg^{-1}; Nanjing Chemlin Chemical Industry, Nanjing, China).or vehicle (0.9% methylcellulose) for 3 days. Mice were killed by decapitation at different times after paracetamol administration, and blood and liver tissue were collected.

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) assays

Blood was held at 4°C for 1 hr, then centrifuged at 3000 x *g* for 10 min. Serum was collected from the supernatant and stored at -80°C. Serum ALT and AST levels were measured by appropriate enzymic kits (Zhongsheng Technologies, Beijing, China) by the National Chengdu Center for Safety Evaluation of Drugs.

Liver GSH/GSSG assay

Hepatic non-protein thiol levels were measured as an indicator of hepatic GSH content. At the times shown after paracetamol treatment, livers were removed and immediately homogenized in 5% trichloroacetic acid, then centrifuged at 1000 x *g* for 10 min. The supernatant was used to detect liver GSH/GSSG ratio by following the instructions of the Nanjing Jiancheng Bioengineering Institute (China). Briefly, the supernatant was mixed with 10mM 5, 5′-dithio-bis (2-nitrobenzoic acid) and absorbance was measured at 412 nm within 5 min. Reduced glutathione (GSH) was used to generate a standard curve.

Histological staining

The left lobe of livers was removed and immediately fixed in 4% formaldehyde solution, embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin (H&E). Samples were examined under a light microscope, at 200X; magnification.

Detection of liver H_2O_2 levels and thiobarbituric acid reactive substances (TBARS) production

H_2O_2 levels in liver were measured by use of the Amplex red H_2O_2 assay kit (Invitrogen, Grand Island, NY) as described by He *et al.* (2013). Liver samples were homogenized on ice in KCl (150 mM) solution, using a Polytron homogenizer. For each assay, 100 μl supernatant was incubated with 900 μl reaction buffer containing 0.67% thiobarbituric acid at 98°C for 1 h, then centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant absorbance was measured at 532 nm with a spectrophotometer, then converted to nmol TBARS using a standard curve generated with 1,1,3,3- tetramethoxypropane.

RT-PCR analysis

Total liver and cellular RNA was extracted by using Trizol reagent (Invitrogen), and 1000 ng RNA was reverse transcribed to cDNA by use of the iScript cDNA synthesis kit (Bio-rad, Hercules, CA). Quantitative real-time PCR involved the CFX96 real time system (Biorad) with SYBR Green Mix (Biorad) or predeveloped Taqman gene expression assay (Applied Biosystems, Foster City, CA). All of the primers used with SYBR green were designed to span at least one exon to minimize the possibility of non-specific amplification from genomic DNA. The expression of 18s gene (for animal samples) or cyclophilin (for cellular samples) was used as a housekeeping gene to normalize data. Specific primer sequences are in supplementary table S1. Amplification specificity was evaluated by determining the product melting curve and involved the following program: 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 20 s and elongation at 72°C for 30 s.

Data analysis

Quantitative results are expressed as the mean±SEM. Experiments were repeated at least 3 times with similar results. Statistical significance was determined by Student's unpaired two-tailed *t* test, or one-way ANOVA for multiple comparisons with Tukey's *post hoc* test. $P<0.05$ was considered statistically significant.

Results

Inhibition of 5-LO protects mice against paracetamol- induced liver toxicity

We first examined whether deletion of 5-LO had conferred an altered sensitivity to paracetamol toxicity. WT mice or 5-LO$^{-/-}$ mice were given saline or paracetamol (200 mg kg^{-1}) by gavage, after overnight fasting. After saline treatment, serum ALT and AST levels did not differ between 5-LO$^{-/-}$ and WT mice (Figure 1A and B). After paracetamol treatment,

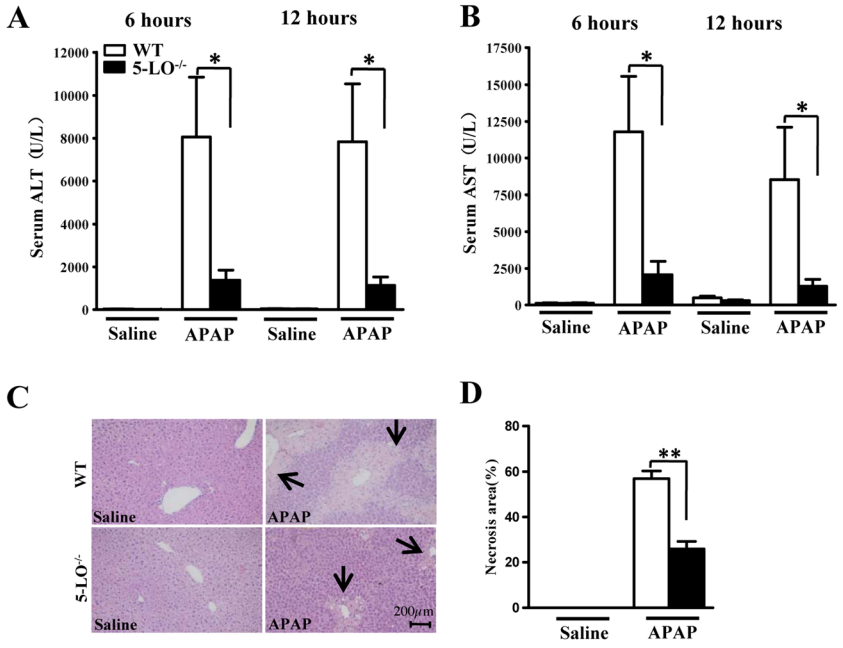

Figure 1

5-LO deletion in mice conferred resistance to paracetamol-induced hepatotoxicity. WT and 5-LO−/− mice fasted overnight then were given oral administered paracetamol (APAP; 200 mg · kg^{-1}). Blood and tissue were collected at the indicated times. Serum levels of (A) ALT and (B) AST in mice treated with saline or paracetamol for 6 or 12 h. (C) Representative H&E staining of liver sections of WT and 5-LO$^{-/-}$ mice. Paracetamol-induced centrilobular necrosis is indicated by arrows. (D) Quantification of liver necrosis area. Data are mean ± SEM from n = 5–11 mice. * P <0.05, ** P <0.01; significantly different as indicated.

the WT liver showed increased serum ALT and AST levels at 6 and 12 h. In contrast, paracetamol-treated 5-LO$^{-/-}$ mice showed greatly reduced ALT and AST levels, compared with WT mice (Figure 1A and B). Consistent with serum ALT and AST levels, WT livers showed the typical necrotic liver damage at 12 h after paracetamol treatment (Figure 1C and D). However, 5-LO$^{-/-}$ mice showed little signs of liver damage (Figure 1C and D), suggesting that deletion of 5-LO conferred resistance to paracetamol hepatotoxicity. The hepatoprotective effect of 5-LO deletion prompted us to determine whether pharmacological inhibition of 5-LO would have a similar effect in preventing paracetamol toxicity. WT C57BL/6J mice were pretreated with vehicle or the 5-LO inhibitor, zileuton, for 3 days before paracetamol administration. Mice were killed 12 h after paracetamol treatment, and blood and liver tissues were collected. Zileuton-treated mice showed significantly reduced serum levels of ALT and AST (Figure 2A and B) and less histological liver damage (Figure 2C and D). However, zileuton has no effect on paracetamol-increased serum ALT and AST levels in 5-LO$^{-/-}$ mice (Supporting Information Fig. 2), indicating zileuton's effect was likely to be mediated by inhibition of 5-LO. These results suggest that inhibition of 5-LO protected mice from paracetamol-induced liver toxicity.

5-LO deletion alters paracetamol-metabolizing enzymes

Resistance to paracetamol toxicity in 5-LO$^{-/-}$ mice suggested that 5-LO deletion may inhibit the formation of toxicity-induced metabolites and/or promote paracetamol clearance. To investigate whether 5-LO signalling affected paracetamol metabolism, we first measured phase I enzymes known to facilitate the formation of toxic paracetamol metabolites. With paracetamol treatment, 5-LO$^{-/-}$ mice showed lower expression of Cyp3a11, whereas the expression of the Cyp1a2 and Cyp2e1 genes remained largely unchanged (Figure 3A–C). Among phase II enzymes, Sult2a1 and glutathione S-transferase (Gst) M1 expression was increased in 5-LO$^{-/-}$ mice after paracetamol administration (Figure 3C and D). The expression of Gst M2, A1 and A2 was unaffected (Supporting Information Fig. 1A–C). Levels of the hepatic transporter MRP3 were significantly decreased in 5-LO$^{-/-}$ mice with paracetamol treatment (Figure 3F), which is consistent with a previous report that Mrp3$^{-/-}$ mice were resistant to paracetamol hepatotoxicity (Aleksunes *et al.*, 2005).

Higher GSH levels in paracetamol-treated 5-LO$^{-/-}$ mice

GSH plays a critical role in neutralizing the reactive intermediate of paracetamol. Baseline GSH levels were similar between WT and 5-LO$^{-/-}$ mice (Figure 4A). With paracetamol treatment, the GSH levels were markedly depleted in the WT at 2 h and gradually recovered at 12 h. GSH levels were higher in 5-LO$^{-/-}$ than WT mice at 2 h with paracetamol treatment (Figure 4B). As a result, the ratio of reduced to GSSG (GSH/GSSG) was higher in 5-LO$^{-/-}$ mice. This finding is consistent with previous reports of GSH depletion correlated with hepatic paracetamol toxicity (James *et al.*, 2003).

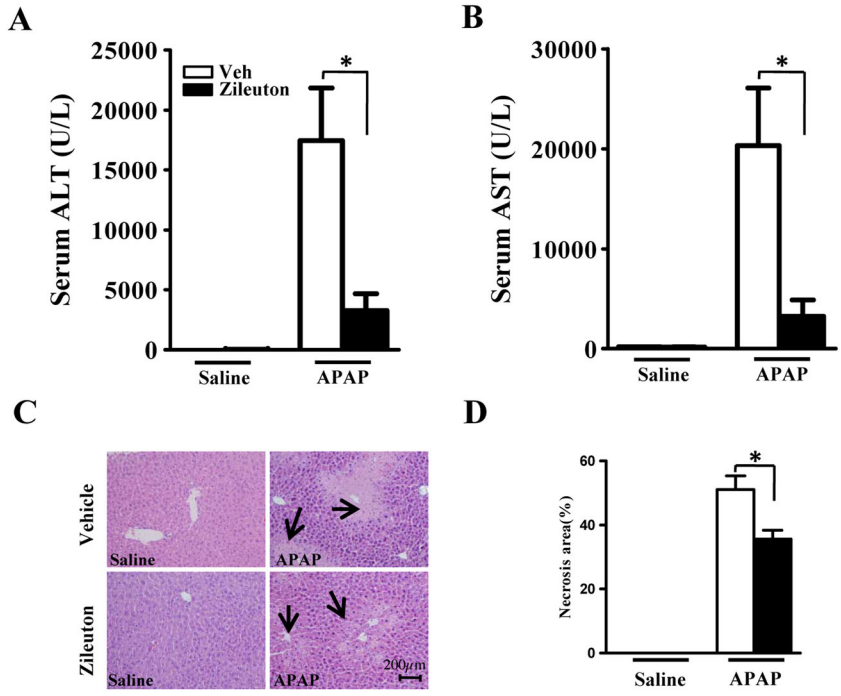

Figure 2

Pharmacological inhibition of 5-LO protected against paracetamol-induced hepatotoxicity. C57BL/6J mice were pretreated with vehicle or zileuton (5-LO inhibitor) for 3 days. At 6 h after paracetamol (APAP) administration, mice were killed, and blood and liver tissue was collected. (A and B) Serum levels of ALT and AST. (B) H&E staining of liver sections from paracetamol-treated or saline-treated mice. Paracetamol-induced centrilobular necrosis is indicated by arrows. (D) Quantification of liver necrosis area. Data are mean ± SEM from $n = 5$–11 mice. * $P < 0.05$; significantly different as indicated.

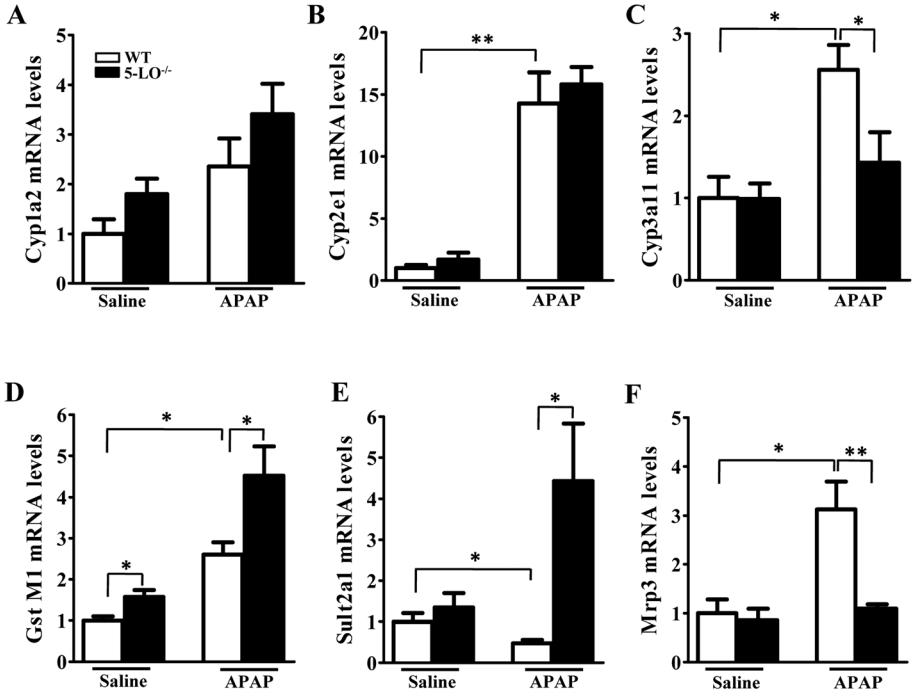

Figure 3

5-LO deletion alters paracetamol-metabolizing enzymes. WT and 5-LO$^{-/-}$ mice were treated with saline or paracetamol (APAP) as described in Figure 1. Real-time PCR analysis of hepatic mRNA expression of phase I, phase II and transporter genes. Data are mean ± SEM from $n = 5$–11 mice for each group. * $P < 0.05$,** $P < 0.01$; significantly different as indicated.

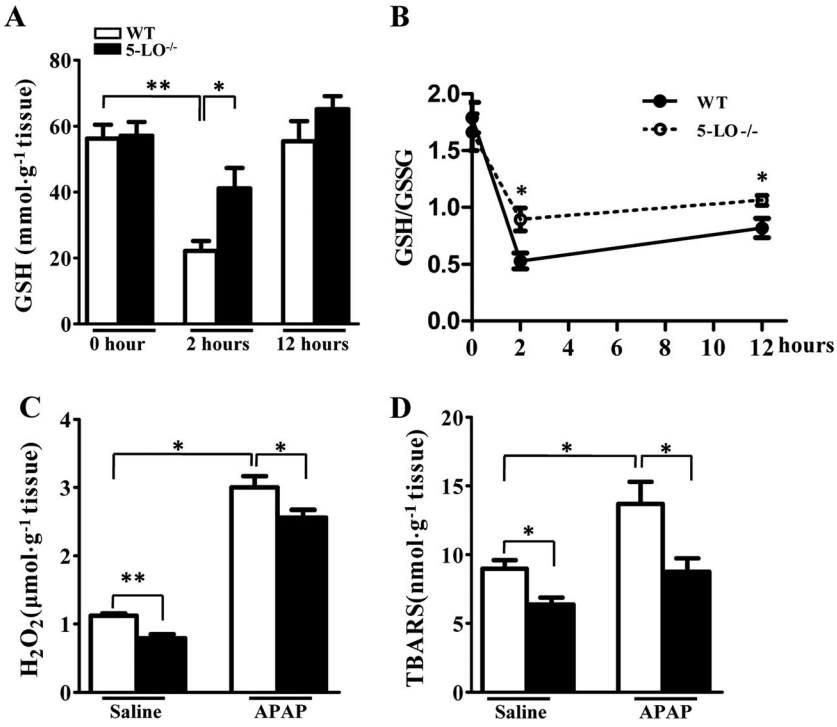

Figure 4

Increased GSH level and reduced ROS production in paracetamol-treated 5-LO$^{-/-}$ mice. WT and 5-LO$^{-/-}$ mice were treated with saline or paracetamol (APAP) as described in Figure 1. (A) GSH level in 5-LO$^{-/-}$ mice with paracetamol treatment. (B) GSH/GSSH ratio. (C) Hepatic H_2O_2 level. (D)Levels of paracetamol-induced TBARS. Data are mean ± SEM from n = 5–11 mice for each group. * P <0.05, ** P <0.01; significantly different as indicated.

5-LO knockout decreased ROS levels in mice

A possible mechanism of paracetamol toxicity is by induced oxidative stress, which may result from depletion of reduced GSH. Paracetamol greatly increased hepatic H_2O_2 levels in both WT and 5-LO$^{-/-}$ mice (Figure 4C). However, 5-LO deletion reduced hepatic H_2O_2 levels, compared with WT mice. This observation agrees with the increased GSH levels at this time in 5-LO$^{-/-}$ mice. The levels of TBARS, a measure of lipid peroxidation, were lower in 5-LO$^{-/-}$ than WT mice, most likely because of reduced H_2O_2 production in livers of 5-LO$^{-/-}$ mice.

5-LO knockout did not alter hepatic inflammation

The 5-LO is known to catalyse the biosynthesis of LTs, lipid mediators of inflammation derived from arachidonic acid (Brock, 2005). Inflammation has been reported to participate in paracetamol-induced hepatic injury (Hinson *et al.*, 2010). Levels of the inflammatory cytokines CCL2, TNF-α and IL-6 were not changed in 5-LO−/− mice (Figure 5A–C), nor was the hepatic expression of CD68, a marker of macrophages (Figure 5D). Meanwhile, 5-LO deletion did not affect the phosphorylation levels of ERK1/2 and JNK1/2, known mediators of inflammation (Figure 5E), as was previously shown (Hinson *et al.*, 2010).

PPARα activation in 5-LO$^{-/-}$ mice

PPARα plays an important role in protecting mice against paracetamol- induced liver injury (Patterson *et al.*, 2012). The expression of PPARα and its target genes Cyp4a10, Cyp4a14, Ucp-2 and Cd36 was greatly increased in 5-LO$^{-/-}$ mice (Figure 6A–E). Previous reports showed that Ucp-2 decreased liver mitochondrial oxidation, which could explain the lower ROS levels and reduced liver injury in 5-LO$^{-/-}$ mice. In contrast, the lipogenic gene, fatty acid synthase, was inhibited in 5-LO$^{-/-}$ mice (Figure 6F), which is consistent with a previous report showing that activation of PPARα inhibited lipogenesis (Iizuka *et al.*, 2013). It seems that PPARα was specifically activated in 5-LO$^{-/-}$ mice, because PPARγ and expression of its target gene FABP4 were not changed by 5-LO or paracetamol treatment (Supporting Information Fig. 3)

Discussion and conclusions

In this study, we showed that inhibition of 5-LO protected mice against paracetamol-induced liver toxicity. Deletion or pharmacological inhibition of 5-LO prevented paracetamol-increased serum ALT and AST values and hepatic necrosis. Mechanistically, 5-LO deletion in paracetamol-treated mice induced the expression of the phase II conjugating enzyme Sult2a1 but decreased that of the P450 enzyme CYP3A11, which metabolizes paracetamol into toxic NAPQI. This protective effect of 5-LO deletion was associated with PPARα activation and reduced ROS production. 5-LO deletion did not affect the inflammatory response with paracetamol overdose.

The majority of paracetamol is either glucuronidated or sulfated and then excreted in urine. In 5-LO$^{-/-}$ mice, Sult2a1

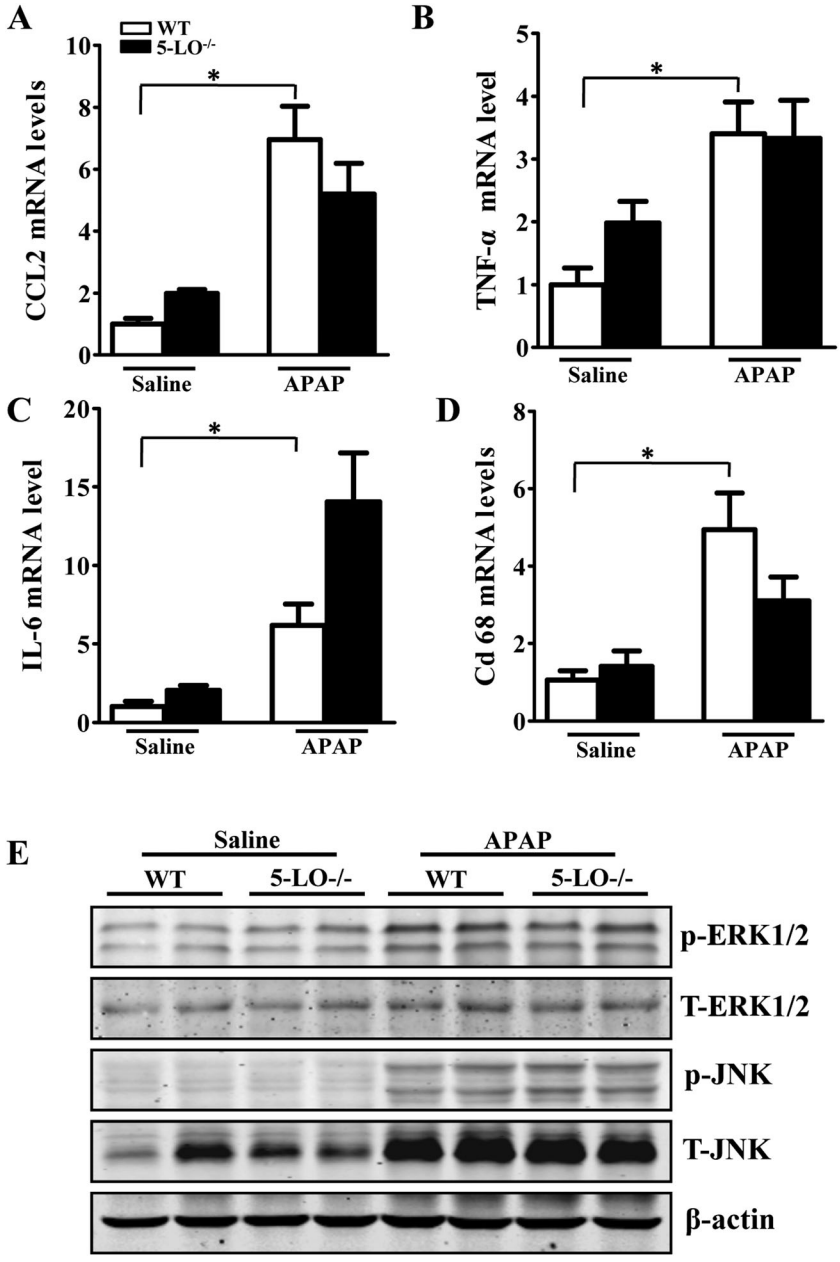

Figure 5

Deletion of 5-LO did not alter hepatic inflammation. WT and 5-LO$^{-/-}$ mice were treated with saline or paracetamol (APAP) as described in Figure 1. (A–C) Real-time PCR analysis of hepatic inflammatory cytokine gene expression. (D) Content of CD68, a marker of macrophages, was not changed between WT and 5-LO$^{-/-}$ mice. (E) Western blot analysis of phosphorylation of ERK and JNK. n = 5–11 mice for each group. * P <0.05; significantly different as indicated.

expression was greatly induced after paracetamol overdose. Increased expression of Sult2a1 has been reported to increase paracetamol urinary clearance and prevent its toxicity (Saini *et al.*, 2011). The metabolic activation of paracetamol is principally catalysed by P450 enzymes, and the reactive metabolite of greatest relevance for hepatotoxicity is generally believed to be NAPQI. The formation of NAPQI was highest with CYP3A4, followed by CYP2E1 and CYP1A2, both at therapeutic and toxic concentrations (Laine *et al.*, 2009). As a CYP3A4 homologous gene, Cyp3a11 plays a key role in metabolizing paracetamol into the toxic NAPQI. The suppression of Cyp3a11 in 5-LO$^{-/-}$ mice could account for the reduced hepatic toxicity as CYP3a11 expression has already been associated with the severity of paracetamol-induced hepatic injury (Guo *et al.*, 2004). The hepatic transporters also play an important role in paracetamol-induced liver toxicity. We found greatly reduced levels of the basolateral transporter MRP3 in 5-LO$^{-/-}$ mice, which is consistent with a previous report that deletion of MRP3 significantly reduced the severity of paracetamol-induced hepatotoxicity (Manautou *et al.*, 2005).

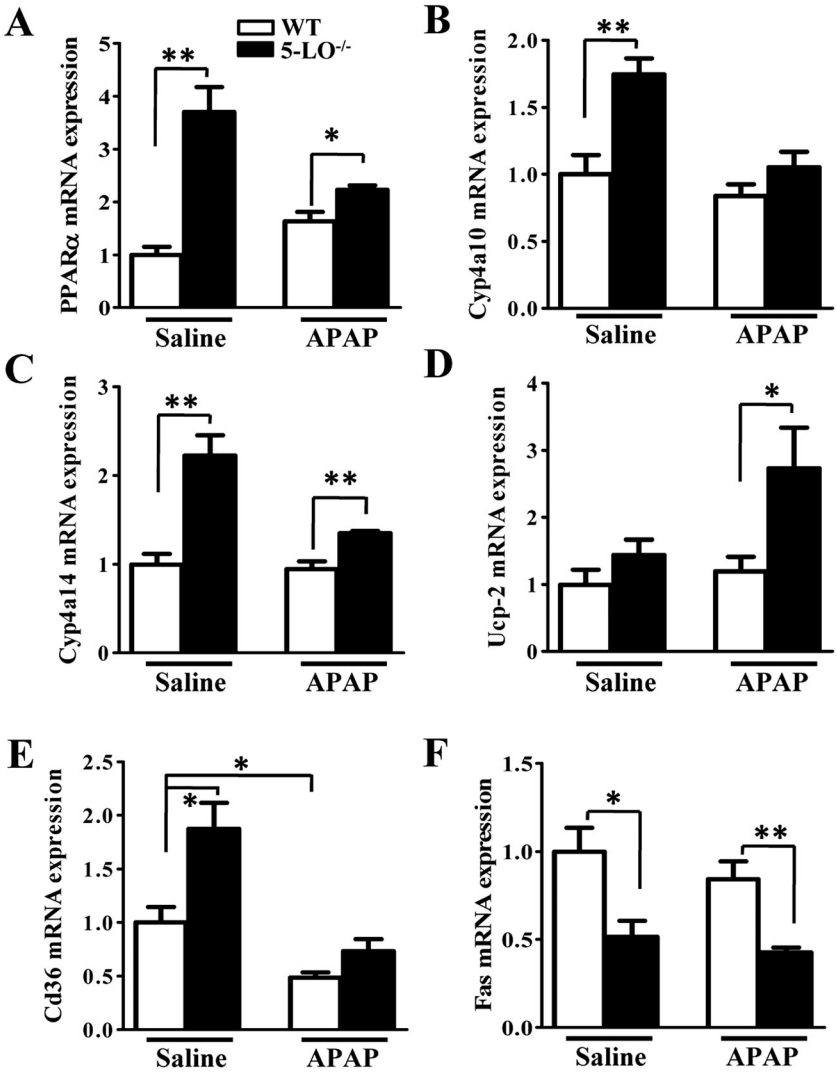

Figure 6

Activation of PPARα in 5-LO$^{-/-}$ mice. Real-time PCR analysis of the expression of PPARα and its target gene by real-time PCR. (A) PPARα and (B–E) target genes in WT and 5-LO$^{-/-}$ mice. (F) Expression of a lipogenic gene, fatty acid synthase. Data are mean ± SEM from $n = 5$–11 mice for each group. * $P < 0.05$, ** $P < 0.01$; significantly different as indicated.

We attribute the reduced paracetamol toxicity in 5-LO$^{-/-}$ mice to an increased level of hepatic GSH, which is important in neutralizing the reactive intermediate of paracetamol. At low doses of paracetamol, GSH efficiently detoxifies paracetamol metabolites by forming a paracetamol–GSH conjugate. However, at high doses, hepatic GSH was depleted, and the paracetamol metabolites covalently bound to protein. GSH depletion and covalent binding are initial events in paracetamol toxicity and lead to oxidative stress, which further exacerbates hepatic toxicity by causing lipid peroxidation and cellular injury (Gautam *et al.*, 2006; Rost *et al.*, 2007; Agarwal *et al.*, 2011). Cellular oxidative stress produced by paracetamol overdose was alleviated in 5-LO$^{-/-}$ mice, as indicated by reduced H_2O_2 level and TBARS concentration. The decreased oxidative stress in 5-LO$^{-/-}$ mice could result from increased level of GSH, a cofactor for GSH peroxidase that prevents peroxide accumulation and decreases oxidative stress via a Fenton mechanism, or 5-LO-generated arachidonic acid metabolites, which induce ROS generation by stimulating NADPH oxidases.

The activation of PPARα in 5-LO$^{-/-}$ mice is intriguing. Arachidonic acid is metabolized primarily by three enzymes: lipoxygenase, COX and cytochrome P450 (Levick *et al.*, 2007). Lipoxygenase enzymes are classified as 5-LO, 8-LO, 12-LO and 15-LO by position of the insertion of oxygen in arachidonic acid (Cho *et al.*, 2011). Among their products, LTB_4 and 8(S)-HETE are endogenous agonists for PPARα, and 12(S)-HETE and 15(S)-HETE activate PPARγ (Muga *et al.*, 2000; Narala *et al.*, 2010; Sun *et al.*, 2015). In the arachidonic acid-metabolizing pathway, inhibition of 5-LO activity is accompanied by increased activity of 12-LO and 15-LO (Vanderhoek *et al.*, 1985; Larsen *et al.*, 2008). 5-LO inhibition might increase the product of the endogenous ligand for PPARα. This suggestion is supported by recent studies showing that a 5-LO inhibitor up-regulated PPARα and its target genes expression (Rodrigues and Machinist, 1996). The induction of

Ucp-2 expression is linked to the protective role of PPARα (Patterson *et al.*, 2012). Overexpression of Ucp-2 protected WT mice against paracetamol-induced hepatotoxicity, while Ucp-2$^{-/-}$ mice were sensitive despite activation of PPARα. Although the induction of Ucp-2 is known to be beneficial, the increased PPARα-promoted fatty acid oxidation might have a chance to increase the overall ROS production. However, fatty acid oxidation is greatly suppressed as a consequence of paracetamol overdose (Chen *et al.*, 2009) This suggests that PPARα-controlled fatty acid oxidation may not be a major source of ROS production. Indeed, activation of PPARα has been shown to decrease ROS production (Patterson *et al.*, 2012), which is consistent with our results (Figure 4C). Taken together, these results suggest PPARα activation could partially explain the beneficial effect of 5-LO deletion in paracetamol overdose. In the future, PPARα$^{-/-}$ mice should be used to further evaluate the contribution of PPARα activation in the beneficial effects of 5-LO deletion.

While this manuscript was in preparation, other authors observed a similar beneficial effect of 5-LO deletion (Hohmann *et al.*, 2013). However, we observed a reduced level of the paracetamol-metabolizing enzyme CYP3A11, increased level of the phase II conjugating enzyme Sult2a1 and decreased level of the hepatic transporter MRP3, which was not evaluated in the Hohmann *et al*. study. Furthermore, in contrast to our study, Hohmann *et al*. found that 5-LO deletion decreased paracetamol-induced liver inflammation. Finally, we found PPARα activated by 5-LO deletion, whereas Hohmann *et al*. showed a slight increase in NRF2 mRNA level. Although different mechanisms were proposed, both studies suggested that 5-LO participated in paracetamol-induced liver toxicity.

In summary, the present study demonstrates that 5-LO inhibition confers resistance to paracetamol- induced hepatotoxicity. With lack of 5-LO, levels of the conjugating enzyme Sult2a1 were increased and that of paracetamol-metabolizing enzyme CYP3A11 was decreased. The protective effect of 5-LO deletion was partially attributed to increased levels of GSH and reduced ROS production. Moreover, levels of PPARα and its target genes were increased in livers from 5-LO$^{-/-}$ mice. Targeted interruption of the 5-LO pathway may be a potential treatment strategy in paracetamol-induced hepatotoxicity.

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Author contributions

S. P. designed and performed experiments and wrote the manuscript. L. R., Q. L., Y. K., J. S., S. C. and Y. Z. helped with experiments. W. J., Z. Z. and C. J. contributed to the discussion and review of the manuscript. J. H. obtained funding, designed experiments and wrote the manuscript. S. P. and J. H. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

The authors have nothing to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Ablation of 5-LO did not alter hepatic Gst M2, A1 and A2 expression. WT and 5-LO$^{-/-}$ mice were treated as described in Fig. 1. **A-C**, Real-time PCR analysis of hepatic mRNA expression of Gst M2, A1 and A2 expression. Data are mean ±SEM from n = 5-11 mice for each group. *$P < 0.05$.
Figure S2 Zileuton has no effect on paracetamol-increased serum ALT and AST levels in absence of 5-LO. 5-LO$^{-/-}$ mice were treated as described in Fig.2. **A** and **B**, serum levels of ALT and AST. Data are mean ±SEM from n = 5 mice for each group.
Figure S3 Expression of PPARγ and its target gene FABP4 expression were not changed by 5-LO or paracetamol treatment. Real-time PCR analysis of the expression of PPARγ and its target gene by real-time PCR. **A,** PPARγ and **B**, target gene FABP4 in WT and 5-LO$^{-/-}$ mice. Data are mean ±SEM from n = 5-11 mice for each group.