The Role of Sulfotransferase 2B1b in Acetaminophen-induced Liver Injury

by

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Overdose of acetaminophen (APAP) is the leading cause of acute liver failure (ALF). Although mechanisms of APAP-induced liver injury are well known, those that affect the progression of APAP-induced liver disease and facilitate liver recovery are less understood. The sulfotransferase 2B1b (SULT2B1b) participates various of liver relative diseases including metabolic syndrome, chronic liver injury and hepatocellular carcinoma. Our previous study showed that SULT2B1b is transcriptional regulated by Hepatic Nuclear Factor 4α (HNF4α), which is essential for liver development and function. However, the importance of SULT2B1b in APAP-induced acute liver injury remains unknown. In this study, we examined the role of SULT2B1b in APAP-induced hepatotoxicity. We showed that hepatic overexpression of SULT2B1b in liver sensitized mice to APAP-induced liver injury, whereas ablation of Sult2B1b conferred resistance to the APAP hepatotoxicity. Indeed, upregulation of Sult2B1b by Hnf4α in wild type mice showed more severe liver injury and this effect was abolished in Sult2B1b knockout mice evidenced by comparable liver damage. Therefore, we conclude that SULT2B1b represents a potential therapeutic target for the prevention and treatment of APAP-induced acute liver injury.

Key Words: sulfotransferase; HNF4α; acetaminophen; liver injury

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

APAP, acetaminophen; **SULT2B1b**, sulfotransferase 2B1b; **HNF4α**, hepatic nuclear factor 4α; **DLI**, drug-induced liver injury; **ALF**, acute liver failure; **UGT**, UDP-glucuronosyltransferase; **SULT**, sulfotransferase; **CYP**, cytochrome P450 enzymes; **NAPQI**, N-acetyl-p-benzoquinone imine; **GSH**, glutathione; **NAC**, N-acetylcysteine; **LXR**, liver X receptor; **DDC**, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **IL-6**, interleukin-6; **IL-1β**, interleukin-1β; **NAFLD**, nonalcoholic fatty liver disease; **NASH**, non-alcoholic steatohepatitis; **HCC**, hepatocellular carcinoma.

PREFACE

I am heartily thankful to my advisor, Dr. Wen Xie, whose encouragement, guidance and support from the initial to the final stage enabled me to obtain a deeper understanding of this project. He coached me in my independent thinking skills and experiment designing skills, and provided me opportunities and excellent suggestions to present this project.

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I would also like to give a special thanks to EXO. Thanks for their songs accompanying me with several long nights.

Lastly, I would like to thank my family members, who supported and encouraged me to chase my dream and are always there whenever I need them.

1.0 INTRODUCTION

1.1 ACETAMINOPHEN IN LIVER INJURY

1.1.1 Epidemiology

Drug-induced liver injury (DLI) and acute liver failure (ALF) remains a major problem in Western societies. Among different etiologies, acetaminophen (APAP) is the leading cause of ALF[1,2]. An epidemiological study suggests that among 308 consecutive patients with liver failure admitted to 1 of 17 referral centers in the United States between 1998 and 2001, 39% are due to APAP overdose[3]. APAP is a widely and commonly used drug to relieve pain and reduce fever. As early as 1955, APAP was first introduced for prescription[4]. By 1960s, reports appear that APAP is associated with liver injury[5]. In the United States, around 50 million people use APAP or APAP-combination products each week[6]. In 2014, the American Association of Poison Control Centers' National Poison Data System reported 67,187 adult and pediatric cases involving APAP alone and 47,588 cases involving APAP in combination with other drugs. There were 996 cases of major APAP toxicity (defined as life threatening or disabling) and 108 cases of APAP-related deaths[7]. Therefore, liver injury resulting from unintentional APAP overdose becomes an urgently issue that must be solved.

1.1.2 APAP metabolism

The initial phases of APAP toxicity were described in Dr. Gillette's laboratory in the 1970s[8]. At therapeutic doses, about 3% of APAP is excreted as prototype via urine. More than 90% of APAP can be rapidly metabolized by Phase II conjugating enzymes, two thirds through glucuronidation by UDP-glucuronosyltransferases (UGTs) and one third through sulfation by sulfotransferases (SULTs) to the nontoxic compounds which are mainly excreted in the urine and bile. There is also 5%-9% of APAP undergoes another bioactivation pathway by Phase I cytochrome P450 enzymes (CYPs), especially CYP2E1, to the highly reactive toxic intermediate metabolite, N-acetyl-p-benzoquinone imine (NAPQI)[9,10]. It has a short half-life and is rapidly eliminated by conjugation with glutathione (GSH)[11]. Then it is excreted via urine as mercapturic acid and cysteine conjugates.

1.1.3 APAP hepatotoxicity

APAP hepatotoxicity is directly related to the dose. At doses of more than 4 g/day, can lead to serious hepatotoxicity. In the event of APAP overdose, the glucuronidation and sulfation pathways are saturated so more amount of APAP is metabolized by CYPs to NAPQI. Excessive NAPQI depletes intracellular GSH. As a result, accumulated NAPQI will bind to cellular proteins and lead to hepatocyte death[11,12].

The degree of hepatic toxicity correlates with GSH availability. GSH depletion contributes to cellular oxidant stress. NAPQI will bind to critical cellular targets, such as mitochondrial proteins. The ultimate result is alteration in calcium homeostasis, mitochondrial dysfunction with ATP depletion, DNA damage, and intracellular protein modification. These events lead to necrotic cell death[13,14].

There are a bunch of factors influencing APAP hepatotoxicity. First of all, the metabolism of APAP depends on age. Children and infants seem to be less susceptible to APAP hepatotoxicity[15]. Since hepatic toxicity is also relative to the activity of the catalyzing enzyme systems, polymorphisms in CYPs play an important role in APAP metabolism and toxicity[16]. Interestingly, people suffered chronic liver injury are no more sensitive to APAP-induced acute liver injury due to liver regeneration[17,18]. Fasting may also enhance APAP toxicity. It is probably because of GSH depletion and CYP2E1 induction during fasting[19]. Alcohol has an effect on APAP hepatotoxicity but it depends on acute or chronic alcohol intake. Acute intake may protect because alcohol competes with APAP for CYPs. But it is different in chronic alcohol intake. CYP2E1 is induced and GSH is depleted by alcohol[14,19]. All of these factors are involved in APAP hepatotoxicity and play important roles.

1.1.4 Treatment approaches for APAP overdose

The main goal of treatment is to prevent or minimize liver injury following APAP overdose. N-acetylcysteine (NAC), a clinical antidote for APAP overdose, is nearly 100% hepatoprotective when it is given within 8 hours after an acute APAP overdose[14,20]. However, NAC has a narrow therapeutic window[21]. NAC may be given orally or intravenously. Although the oral route is simpler, it frequently causes nausea and vomiting and is unpleasant. Additionally, the standard oral regime is 72 hours in duration as compared with about 20 hours intravenously, although this is somewhat arbitrary[22]. Many centers now shorten the duration of oral use by monitoring the serum APAP level and liver enzyme levels. The intravenous route is generally well tolerated, although there is a significant incidence of anaphylactoid reactions. There is no evidence of difference in efficacy between the two routes. NAC also has some therapeutic effect for patients

who present 10 to 24 hours after ingestion, although its efficacy diminishes as the time to treatment increases. Intravenous NAC may be of benefit when rendered as late as 36 to 80 hours in patients[23,24].

In situations where NAC is not available, oral methionine may be an alternative option. Under such rare circumstances where there is no antidotal therapy available, oral-gastric lavage and activated charcoal should be considered given the morbidity and mortality from APAP toxicity in the absence of antidotal therapy. If no other options are available, hemodialysis may be considered as a means of rapidly decreasing the serum APAP concentration in patients presenting soon after an acute APAP overdose, provided hemodialysis can be expeditiously initiated and its benefits outweigh its risks[25,26].

1.2 SULFOTRANFERASE 2B1b IN APAP-INDUCED LIVER INJURY

1.2.1 Cytosolic SULT family

The human cytosolic SULT family consists of encoding 14 different isoforms. The human SULT2 family, also called hydroxysteroid-SULT family, consists of only 2 genes, termed SULT2A1 and SULT2B1. The SULT2 enzymes sulfate a variety of substrates, such as DHEA, pregnenolone and cholesterol[27]. However, they show different tissue distribution, specificities and activities. Based on the length of transcripts, SULT2B1 termed SULT2B1a and SULT2B1b. In transcriptional level, SULT2B1a and SULT2B1b are highly similar. But only SULT2B1b protein can be detected by immunoblot in human[28]. Therefore, SULT2B1a message may not be efficiently translated in human tissues. SULT2B1b has been identified by immunoblot in prostate[29], placenta[30], intestine[31],

breast[32], skin[33] and platelets[34]. SULT2B1b appears to be the major active SULT2B1 isoform in human tissues. SULT2B1b is selective for the sulfation of 3 -hydroxysteroids and also has activity with cholesterol suggesting it may have different enzymatic functions in different human tissues[27].

1.2.2 SULT2B1b in liver diseases

Previous studies have suggested a critical role of SULT2B1b in regulating the chemical and functional homeostasis of endogenous and exogenous molecules and in a variate of diseases. Based on our previous study, SULT2B1b is transcriptional regulated by HNF4α to prevent uncontrolled gluconeogenesis[35]. Although SULT2B1b expression is fairly low in liver, there have been several reports indicating that SULT2B1b is crucial in regulation of liver function and diseases. SULT2B1 is transcriptionally upregulated during liver regeneration in a mouse model of partial hepatectomy[36]. SULT2B1b mRNA levels in clinical hepatocarcinoma tumor samples were higher than in the non-tumorous tissue adjacent to the tumors[37]. Oxysterols, which are considered as activators of Liver X Receptor (LXR), are also substrates of SULT2B1b[38,39]. Upregulation of SULT2B1b aggravated 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver injury by modulating oxysterol-induced LXR activation[40]. SULT2B1b also can be regulated by nuclear receptors, such as CAR[41], VDR[42] and PPARs[43].

1.3 HNF4A IN APAP-INDUCED LIVER INJURY

Hepatocyte Nuclear Factor 4α (HNF4α) is known to modulate regulatory elements in the promoters and enhancers of genes involved in cholesterol, fatty acid and glucose metabolism[44].

Specifically in the liver, HNF4α activates hepatic gluconeogenesis and regulates the expression of several genes. A recent study by Lake et al. assessed the role of major transcription factor binding sites in non-alcoholic steatohepatitis (NASH)[45]. HNF4α mRNA expression was significantly decreased in human NASH samples, suggesting the contribution of HNF4α to nonalcoholic fatty liver disease (NAFLD) development. Using knockout models, previous mouse studies have revealed the critical role of HNF4α in the control of bile acid synthesis and glucose homeostasis[46,47]. In addition, an integrative analysis of NAFLD signatures in human and genetically modified mouse models demonstrated that HNF4α as a transcription factor plays an important role in regulating the expression of the genes involved in the progression of NAFLD to hepatocellular carcinoma[48].

In this study, we demonstrated that overexpress SULT2B1b in liver sensitized mice to APAP-induced liver injury while deletion of SULT2B1b attenuated its toxicity. Upregulation of SULT2B1b by HNF4α aggravated APAP hepatotoxicity and this effect could be abolished in SULT2B1b knockout mice. Our study pointed to SULT2B1b as a potential therapeutic target for APAP-induced acute liver injury.

2.0 METHODS AND MATERIALS

Animals

The whole-body Sult2B1b knockout mice (Strain # 018773) and C57BL/6J wild type mice were purchased from the Jackson Laboratory (Bar Harbor, ME). FABP-SULT2B1b transgenic mice were bred by our laboratory as previously described[46]. All mice used for the experiments were 6-8 week-old female mice. All mice were housed under a standard 12-hour light and 12-hour dark cycle with free access to food and water. The use of mice in this study complied with all relevant federal guidelines and institutional policies.

Induction of liver injury

APAP was dissolved in 0.5% methyl cellulose solution. All experiments were performed by fasting mice at 4:00 pm and treating APAP to mice by gavage at 8:00 am. Food back 3 hours after treatment. The mice were sacrificed 24 hours after APAP treatment[49]. Liver tissues and serum were harvested for biochemical and histological analysis. All chemicals were purchased from Sigma (St. Louis, MO).

Histology

For H&E staining, tissues were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned at 4 mm, and stained with H&E. For immunohistochemistry analysis, standard immunohistochemical procedures were performed in detection of Ki67 from Abcam (Cambridge, MA). A Novus (Littleton, CO) APO-BRDU (TUNEL) Apoptosis kit was used to examine dying cells with exposed or fragmented DNA ends as per the manufacturer's instruction.

Serum and liver tissue chemistry

Levels of ALT and AST in serum[50] and cells[51] were measured using commercial assay kits from Stanbio Laboratory (Boerne, TX). The concentration of GSH in the liver was measured by GSH Assay Kit from BioAssay Systems (Hayward, CA).

Real-time PCR

Total RNA was isolated using the TRIzol reagent from Invitrogen. Reverse transcription was performed with random hexamer primers and Superscript RT III enzyme from Invitrogen. SYBR Green-based real-time polymerase chain reaction (PCR) was performed with the ABI 7300 Real-Time PCR System. The PCR primer sequences are shown in Table 1. The quantity of mRNA was normalized to the cyclophilin gene.

Western blot

For Western blot analysis, tissues and cells were lysed in ice-cold Nonidet P-40 lysis buffer containing a protease inhibitor cocktail from Roche, and then quantified for protein concentrations by a bicinchoninic acid assay kit from Pierce. Protein samples were resolved by electrophoresis on 10% SDS-polyacrylamide gels. After transfer of proteins to nitrocellulose membranes, the membranes were probed with primary antibodies against total AKT (cat. no. 9272) and phospho-Akt (serine 473) (cat. no. 9271) from Cell Signaling (Beverly, MA). Detection was achieved by using the enhanced chemiluminescence system from Amersham (Piscataway, NJ). The signals were quantified by using the Image J software (http://imagej.nih.gov/ij/).

Isolation and culture of primary hepatocytes from mice

Primary mouse hepatocytes were isolated from 8 to 12 week-old mice, as previously described. Briefly, the liver was first perfused with Hanks' buffered salt solution containing 0.5 mM EGTA and 0.1 M HEPES at 5 ml/min for 5–10 minutes and then perfused with L-15 medium containing 1.8 mM $CaCl_2$, 0.1 M HEPES, and 20 mg/ml liberase (Roche, Indianapolis, IN). After perfusion, the dissociated hepatocytes were filtered through 50-mm pore mesh and collected by centrifugation at 500 rpm for 3 minutes at 4°C. Hepatocytes were seeded onto type 1 collagen-coated dishes in William E medium containing 5% fetal bovine serum. The medium was changed to HepatoZYME-SFM medium (GIBCO, Grand Island, NY) 2 hours later.

Adenovirus transfection

The primary hepatocytes were treated with adenovirus to overexpress Hnf4α. Adenovirus expressing Hnf4α (Ad-Hnf4α) was gift from Dr. Yanqiao Zhang from the Northeast Ohio Medical University.

Statistical analysis

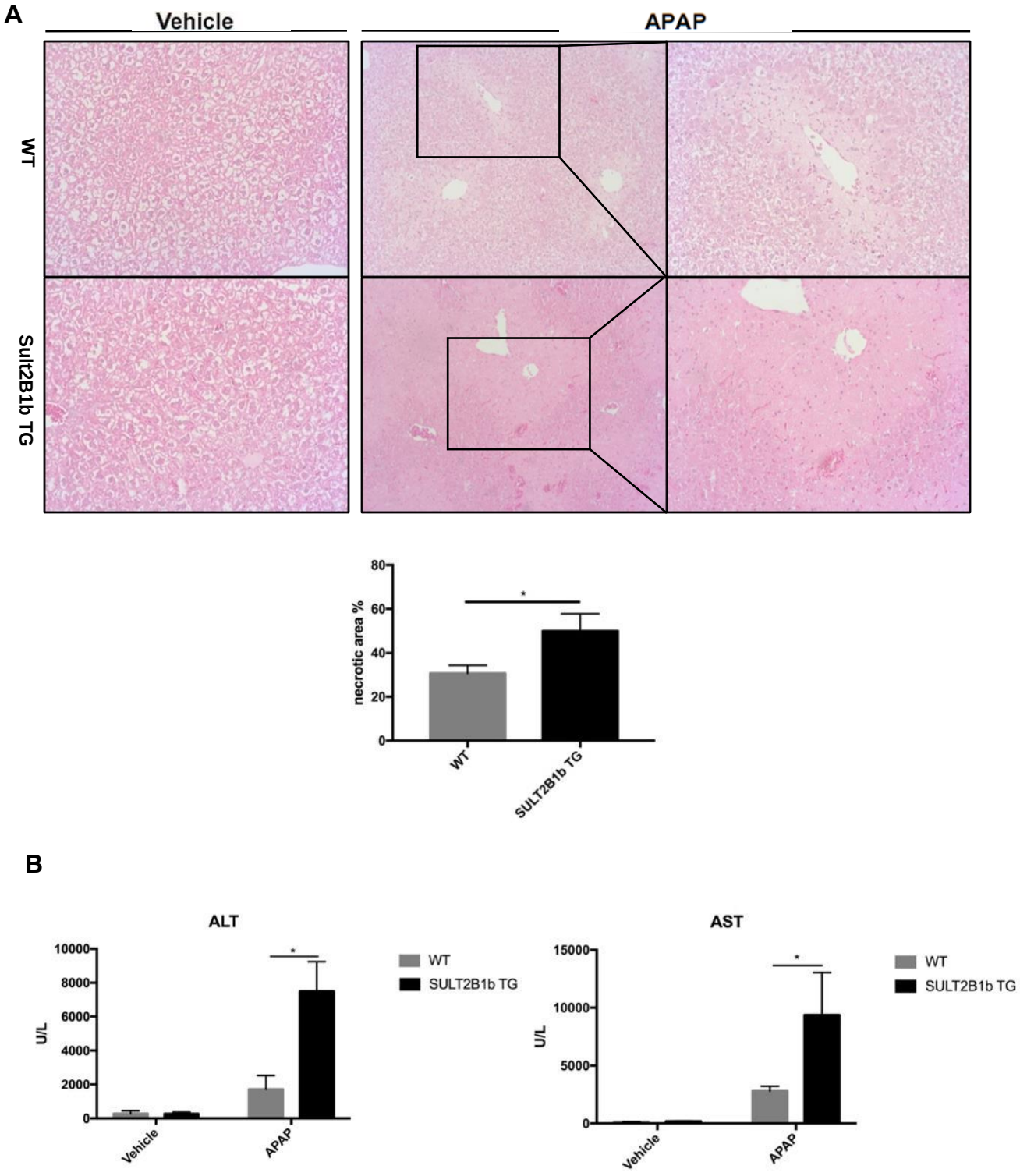
All the data are expressed as means ± standard error of the mean (SEM). Differences were evaluated by the unpaired two-tailed Student's *t* test (GraphPad Prism). The criterion for statistical significance was a *P* value of < 0.05.

3.0 RESULTS

3.1 OVEREXPRESSION OF SULT2B1B IN LIVER AGGRAVATED APAP-INDUCED LIVER INJURY

To investigate whether SULT2B1b is indeed important in the pathogenesis of APAP-induced liver injury, we first treated SULT2B1b transgenic (TG) mice, which specific overexpress SULT2B1b in liver, with a single dose of APAP (200mg/kg, gavage). At 24 hours post-treatment, the tissues and serum were harvested to analysis. H&E staining revealed that the SULT2B1b TG mice displayed more severe damage compared to wild type (WT) mice. In vehicle-treated mice, no significant alteration in liver histology was found in TG mice, when compared to WT mice. But after APAP treatment, TG mice showed more typical necrotic liver damage than WT mice. The ratio of necrotic area in TG mice is higher than in WT mice as well (Fig. 1A). In addition, APAP-treated TG mice showed increased serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, compared to WT mice (Fig. 1B). The extent of hepatocyte DNA fragmentation, as assessed by TUNEL staining, was significantly increased in liver sections from TG mice after APAP treatment (Fig. 1C). Liver regeneration is known for survival after APAP overdose. Thereby, we also evaluated the proliferation and cell cycle-related gene expressions in livers by real-time PCR. TG mice showed significantly higher expression of *Pcna* than WT mice (Fig. 1D). It indicated that TG mice had more liver damage than WT mice. Furthermore, hepatic expression of inflammatory cytokines interleukin-6 (Il-6) and interleukin-1β (Il-1β) in TG mice was much higher than that in WT mice after APAP treatment. (Fig. 1E). Collectively,

overexpression of SULT2B1b results in enhanced liver injury with severe necrosis and increased liver regeneration and inflammation.



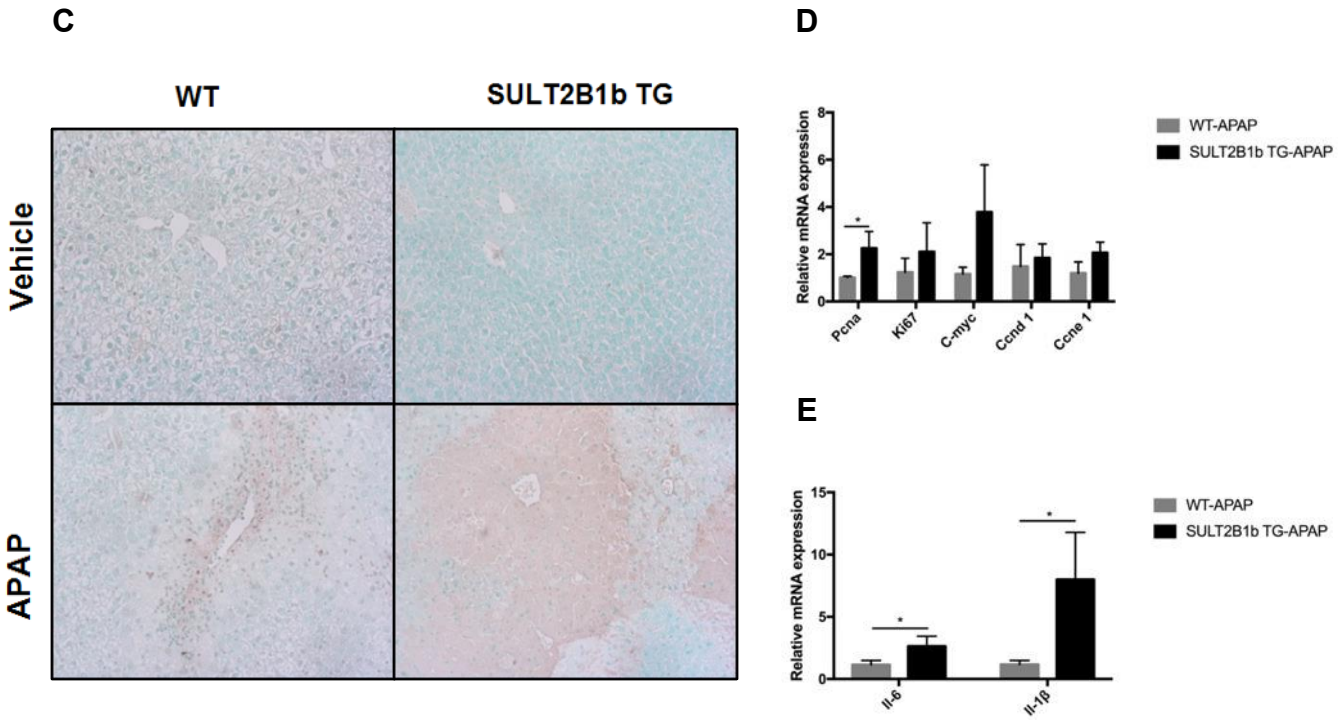
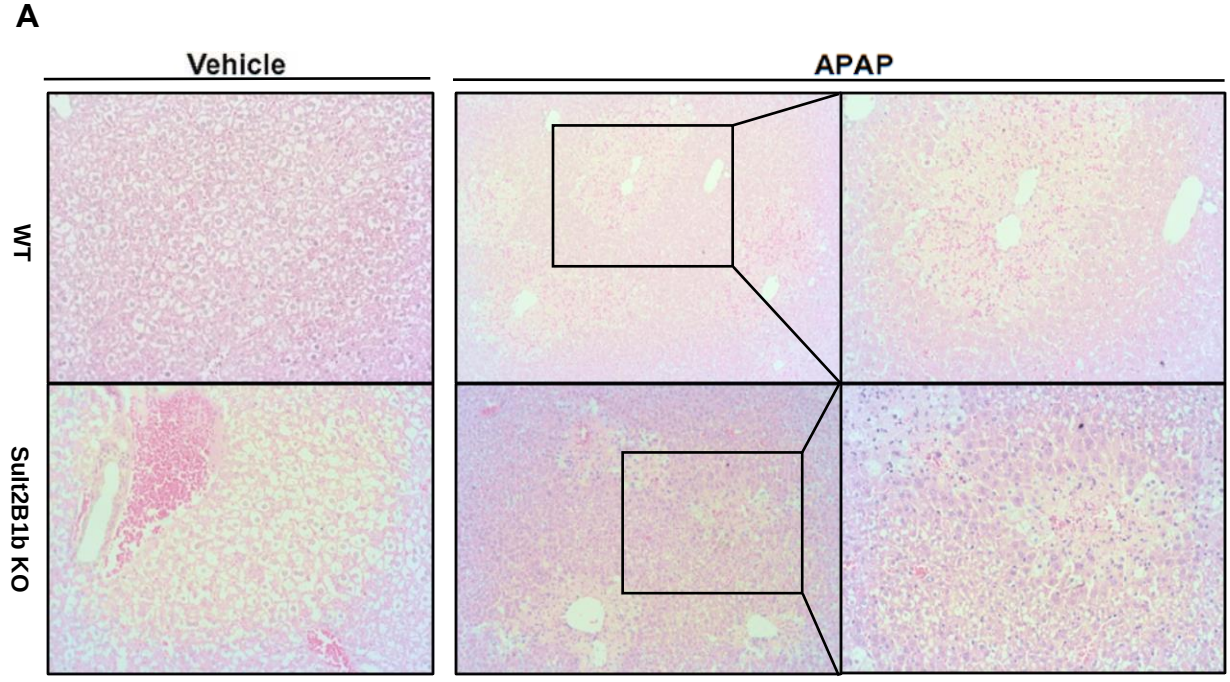
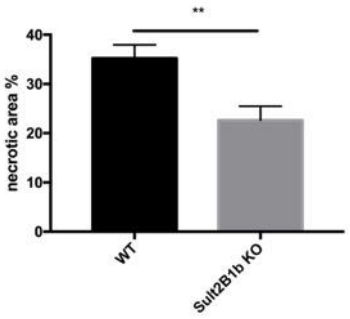


Figure 1. Overexpression of SULT2B1b in liver aggravated APAP-induced liver injury.
A Representative H&E staining on liver paraffin sections at 24 hours after APAP-treated WT and SULT2B1b TG mice (middle, original magnification ×100), APAP-induced centrilobular necrosis (right panels, enlarged view of boxed region in middle panels) and quantification of necrotic areas in liver sections (n=5 per group). **B** Serum levels of ALT and AST in WT and TG mice treated with vehicle or APAP. **C** Representative images of TUNEL staining in liver sections from WT and TG mice at 24 hours after vehicle or APAP treatment (magnification ×200). **D** Relative mRNA levels of *Pcna*, *Ki67*, *C-myc*, *Ccnd1*, and *Ccne1* in the livers. **E** Relative mRNA levels of *Il-6*, *Il-1β* in livers. Data are expressed as mean ± SEM. **P* < 0.05.

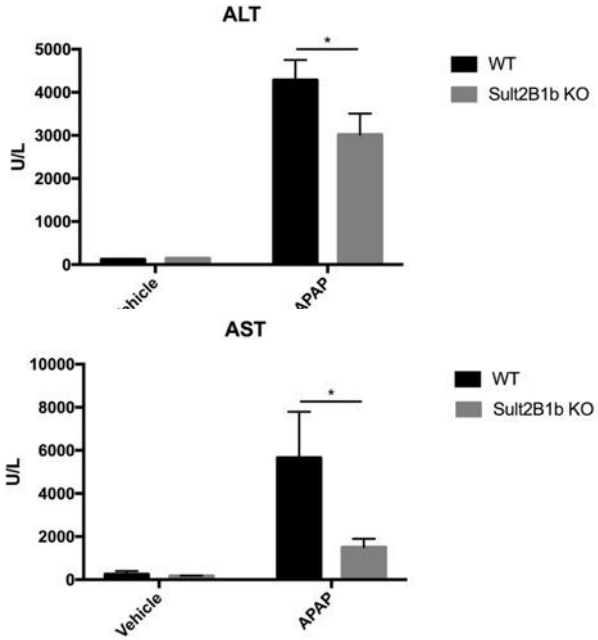
3.2 DELETION OF SULT2B1B PROTECTED MICE FROM APAP-INDUCED LIVER INJURY

To assess whether SULT2B1b ablation affects APAP-induced liver injury, we also treated Sult2B1b knockout (KO) mice with a single dose of 200mg/kg APAP. As a result, Sult2B1b KO mice showed less damage compared to WT mice. The WT liver showed expected typical necrotic liver damage at 24 hours post-APAP. In contrast, KO mice showed little signs of damage (Fig. 2A). Also, APAP-treated KO mice showed reduced serum levels of ALT and AST, compared to their WT counterpart (Fig. 2B). In TUNEL staining, KO mice showed significantly less positive cells (Fig. 2C). We also evaluated proliferation of hepatocytes by Ki67 staining. KO mice showed remarkably less hepatocyte proliferation than WT mice (Fig. 2D). Consistently, mRNA levels of *Pcna* and *Ccnd 1* expressions decreased in KO mice as well (Fig. 2E). Moreover, *Il-1β* mRNA expressions were dramatically induced in WT mice than that in KO mice (Fig. 2F). These results suggest that deletion of Sult2B1b conferred resistance to APAP hepatotoxicity.

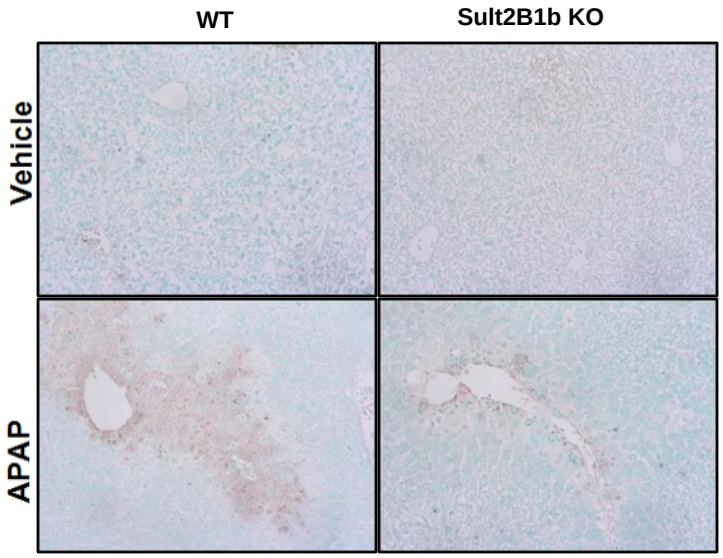



**
necrotic area %
40
30
20
10
0
WT
Sult2B1b KO

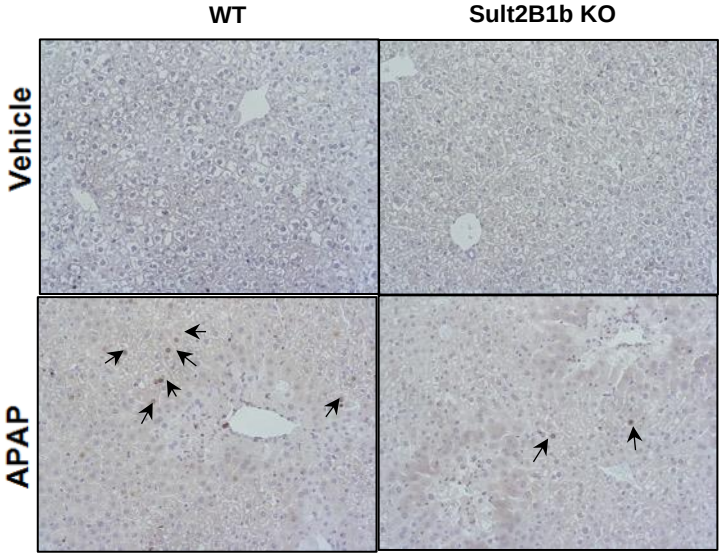
B


ALT
*
U/L
5000
4000
3000
2000
1000
0
WT
Sult2B1b KO
AST
*
U/L
10000
8000
6000
4000
2000
0
WT
Sult2B1b KO
Vehicle
APAP

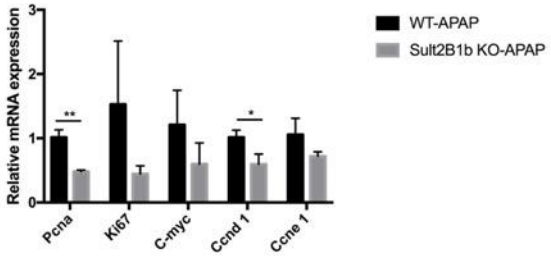
C


WT
Sult2B1b KO
Vehicle
APAP

D


WT
Sult2B1b KO
Vehicle
APAP

E


Relative mRNA expression
3
2
1
0
**
*
WT-APAP
Sult2B1b KO-APAP
Pcna
Ki67
C-myc
Ccnd 1
Ccne 1

F

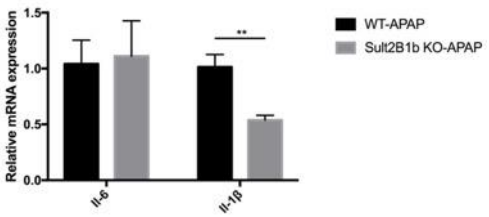

Relative mRNA expression
1.5
1.0
0.5
0.0
**
WT-APAP
Sult2B1b KO-APAP
Il-6
Il-1β

Figure 2. Deletion of Sult2B1b protected mice from APAP-induced liver injury.
A Representative H&E staining on liver paraffin sections at 24 hours after vehicle or APAP-treated WT and Sult2B1b KO mice (middle, original magnification ×100), APAP-induced centrilobular necrosis (right, enlarged view of boxed region in middle panels) and quantification of necrotic areas in liver sections (n=2 for WT-vehicle and Sult2B1b KO-vehicle, n=8 for WT-APAP and Sult2B1b KO-APAP). **B** Serum levels of ALT and AST in WT and KO mice treated with vehicle or APAP. **C** Representative images of TUNEL staining in liver sections from WT and Sult2B1b KO mice at 24 hours after vehicle or APAP treatment (original magnification ×200). **D** Representative images of Ki67 staining in liver sections from WT and Sult2B1b KO mice at 24 hours after vehicle or APAP treatment (original magnification ×200). **E** Relative mRNA levels of *Pcna*, *Ki67*, *C-myc*, *Ccnd1*, and *Ccne1* in the livers. **F** Relative mRNA levels of *Il-6*, *Il-1β* in livers. Data are expressed as mean ± SEM. **P* < 0.05; ***P* < 0.01.

3.3 UPREGULATION OF SULT2B1B BY HNF4A SENSITIZED MICE TO APAP-INDUCED LIVER INJURY

To study the effects of upregulation of SULT2B1b by overexpressing HNF4α on APAP-induced acute liver injury, we injected adenovirus to overexpress Hnf4α in WT mice. After a week, these mice were treated with APAP as previous experiments. Hnf4α and Sult2B1b protein levels were increased compared to control mice (Fig. 3A). Compared to WT mice injected with Ad-Ctrl, WT mice injected with Ad-Hnf4α displayed more severe injured liver (Fig. 3B). H&E staining revealed more severe and widespread necrosis in WT mice with Ad-Hnf4α compared to control mice (Fig. 3B). Liver injury was also assessed by ALT and AST levels. After APAP treatment, ALT and AST levels were significantly higher in WT mice injected with Ad-Hnf4α than in WT mice injected with Ad-Ctrl (Fig. 3C). To further confirm it in vitro, we also isolated primary hepatocytes from WT mice and treated them with Ad-Hnf4α. After 5nM APAP treatment for 24 hours, the percentage of ALT release slightly increased by Ad-Hnf4α (Fig. 3D). These observations suggest that overexpression of HNF4α upregulates SULT2B1b expression and aggravates APAP-induced liver injury.

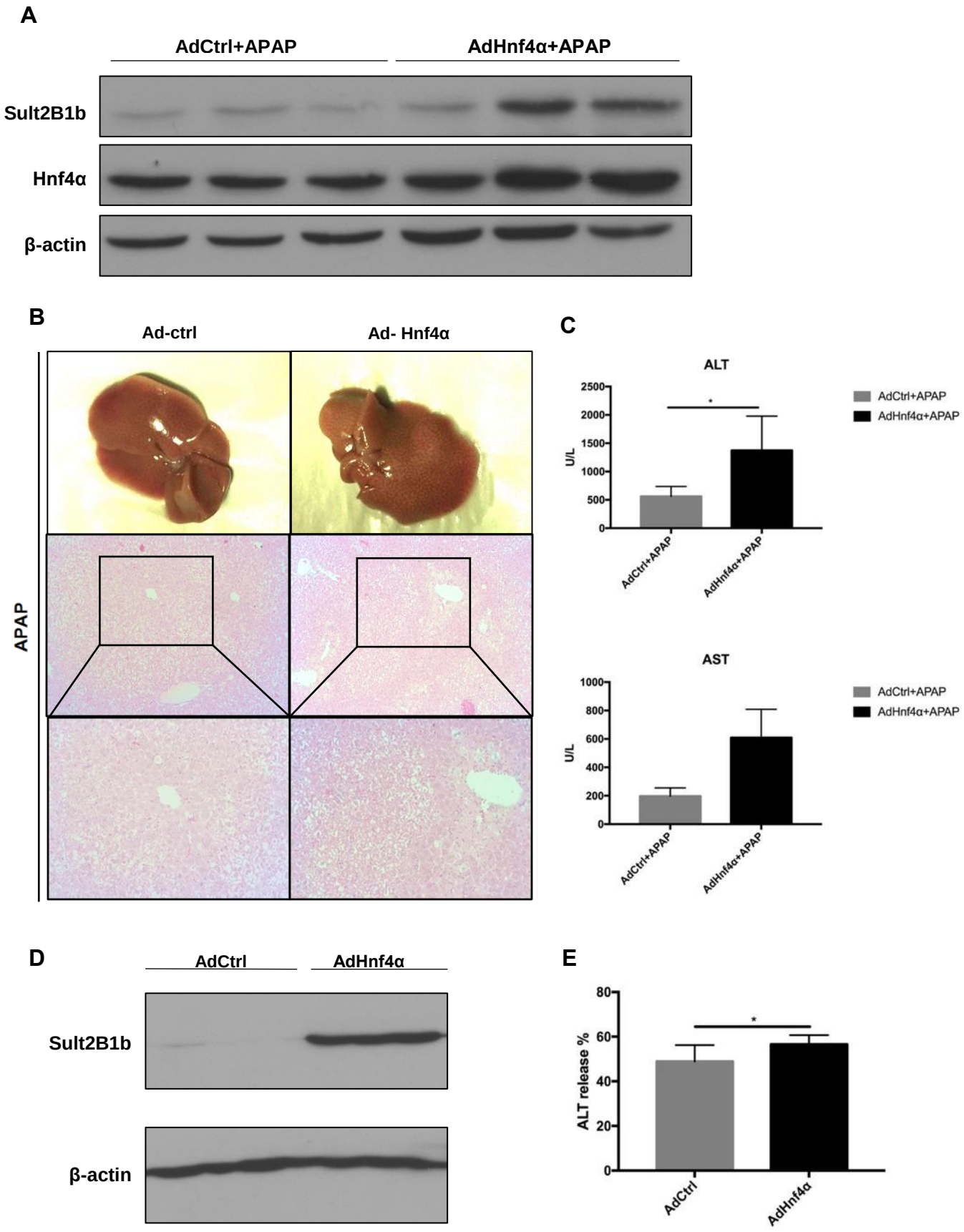

A
AdCtrl+APAP
AdHnf4α+APAP
Sult2B1b
Hnf4α
β-actin
B
Ad-ctrl
Ad- Hnf4α
APAP
C
ALT
U/L
2500
2000
1500
1000
500
0
*
AdCtrl+APAP
AdHnf4α+APAP
AST
U/L
1000
800
600
400
200
0
D
AdCtrl
AdHnf4α
Sult2B1b
β-actin
E
ALT release %
80
60
40
20
0
*
AdCtrl
AdHnf4α

Figure 3. Upregulation of Sult2B1b by Hnf4α sensitized mice to APAP-induced liver injury. **A** Hepatic Sult2B1b and Hnf4α expressions in Ad-ctrl+APAP and Ad-Hnf4+APAP WT mice by Western blot analysis. **B** Representative appearance (top) and H&E staining on liver paraffin sections of Ad-ctrl+APAP and Ad-Hnf4+APAP WT mice (original magnification ×100, middle) and APAP-induced centrilobular necrosis (bottom, enlarged view of boxed region in middle panels). n=3 for Ad-ctrl+APAP, n=5 for Ad-Hnf4+APAP. **C** After APAP treatment, serum levels of ALT and AST in WT mice injected with Ad-ctrl or Ad-Hnf4α. **D** Sult2B1b and Hnf4α expressions in WT primary hepatocytes treated with Ad-ctrl or Ad-Hnf4α by Western blot analysis. **E** The percentage of ALT release from WT primary hepatocytes treated with 5nM APAP for 24 hours. Data are expressed as mean ± SEM. **P* < 0.05.

3.4 SULT2B1B IS ESSENTIAL FOR THE SENSITIZATION OF HNF4A IN APAP HEPATOTOXICITY

To understand whether HNF4α sensitizes mice to APAP hepatotoxicity in a SULT2B1b-dependent manner, we then injected Ad-Ctrl and Ad-Hnf4α respectively to Sult2B1b KO mice. We first verified that Hnf4α was overexpressed in livers of Sult2B1b KO mice (Fig. 4A). After APAP treatment, on the contrary, KO mice injected with Ad-Ctrl and Ad-Hnf4α showed similar levels of ALT and AST (Fig. 4B). The livers showed comparable damage and the histology is consistent with serum analysis as well (Fig. 4C). These findings suggest that deletion of Sult2B1b abolished the effect of Hnf4α overexpression on APAP hepatotoxicity. Therefore, we proved that the sensitization of HNF4α in APAP-induced liver injury is dependent on SULT2B1b.

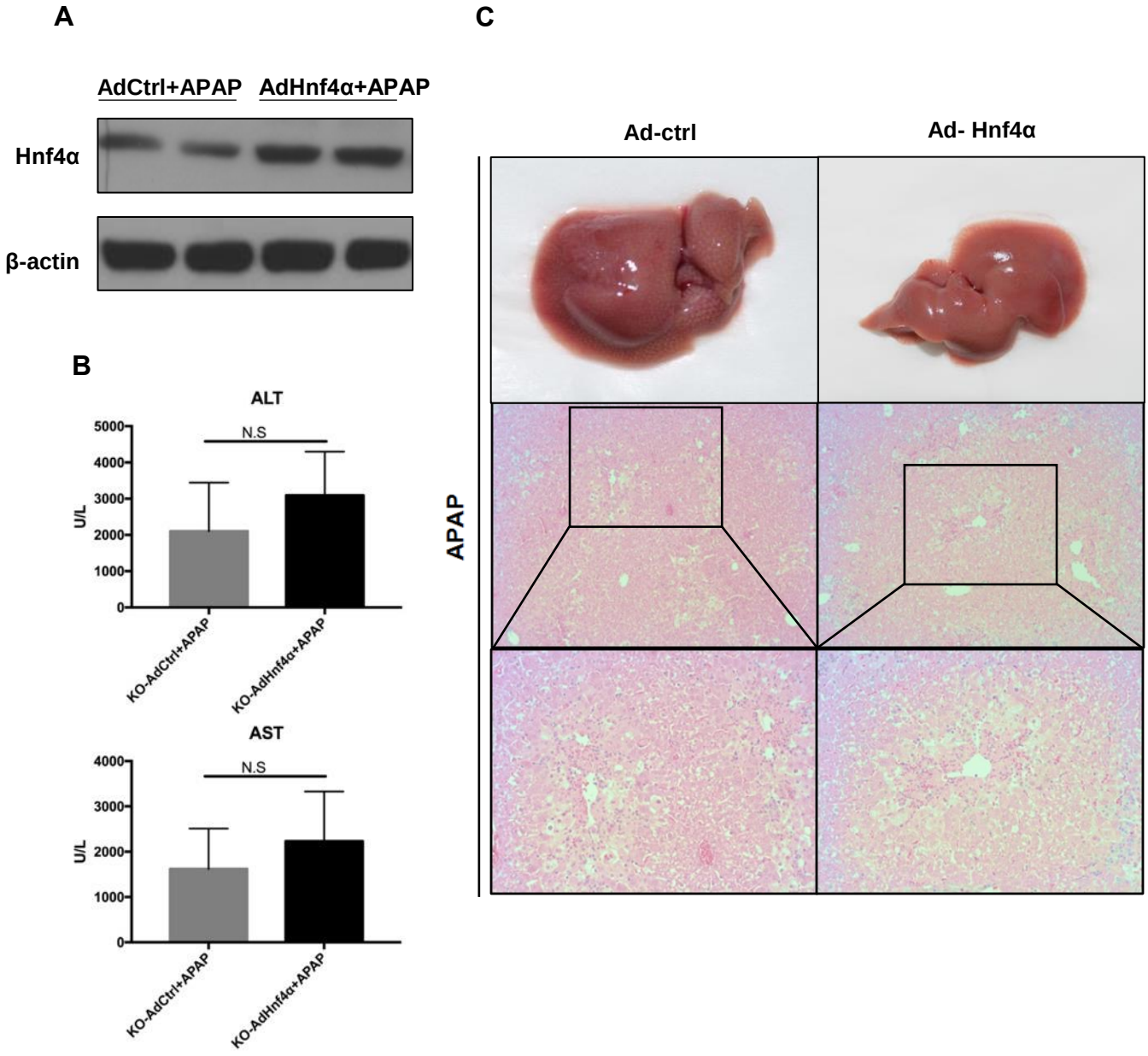
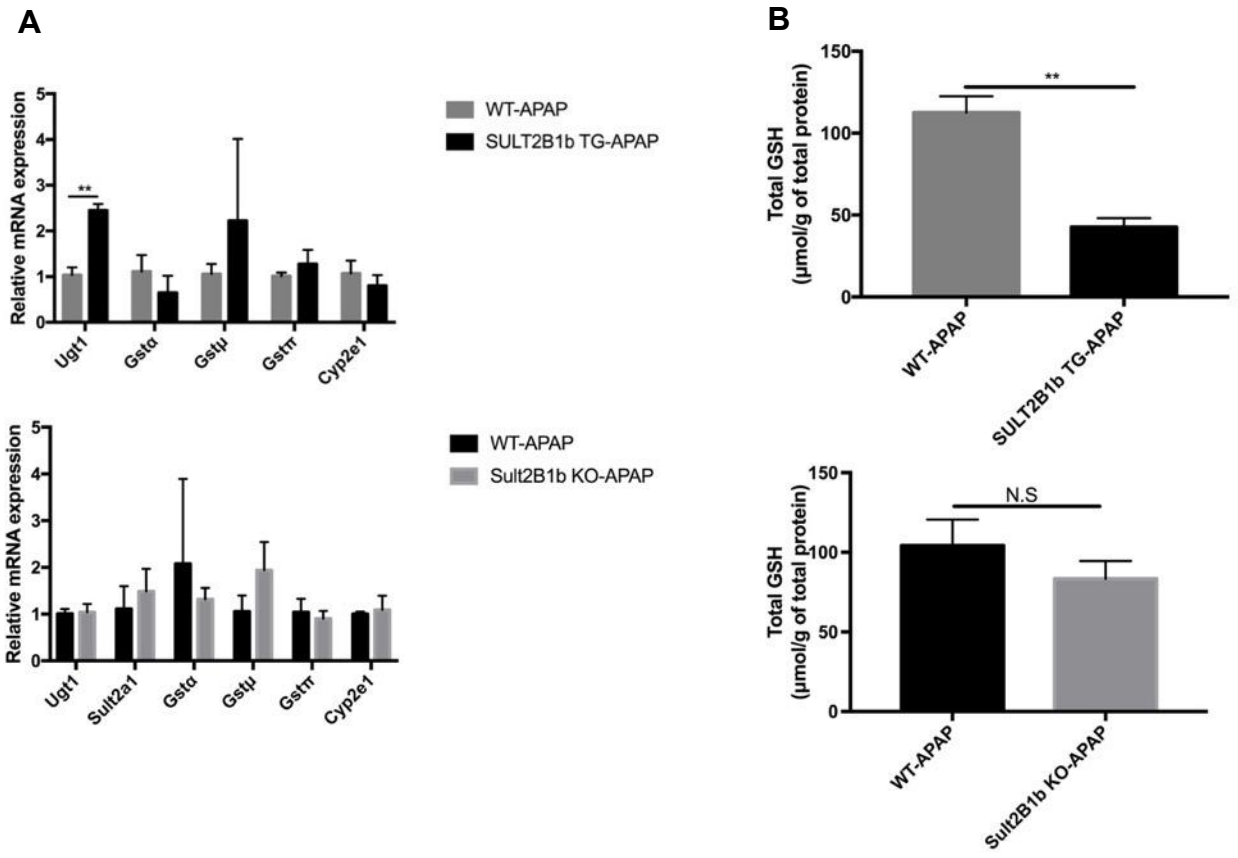


Figure 4. Sult2B1b is essential for the sensitization of Hnf4α in APAP hepatotoxicity.
A Hepatic Hnf4α expressions in Ad-ctrl+APAP and Ad-Hnf4+APAP KO mice by Western blot analysis. **B** Representative appearance (top) and H&E staining on liver paraffin sections at APAP-treated KO mice with Ad-ctrl or Ad-Hnf4α injection (middle, original magnification ×100) and APAP-induced centrilobular necrosis (bottom, enlarged view of boxed region in middle panels). n=3 for Ad-ctrl+APAP, n=5 for Ad-Hnf4+APAP. **C** After APAP treatment, serum levels of ALT and AST in Sult2B1b KO mice injected with Ad-ctrl or Ad-Hnf4α. Data are expressed as mean ± SEM. **P* < 0.05.

3.5 PROFILE OF APAP METABOLISM IN SULT2B1B TG AND KO MICE

To pursue the role of SUT2B1b in APAP metabolism, we compared the difference in APAP metabolism among these mice in different genotypes. Only Ugt1 was induced in TG mice and the expressions of other relative APAP metabolizing enzymes showed no significant difference in both TG and KO mice (Fig. 5A). Then the total hepatic GSH content in TG and KO mice was measured (Fig. 5B). It was significantly reduced in TG mice but no alterations in KO mice. By LC-MS, the concentration of APAP metabolites, APAP-sulfate and APAP-glucuronide, in serum were measured (Fig. 5C). Based on current results, it is limited to make a conclusion, so we will also measure APAP-cysteine content in liver of these mice.



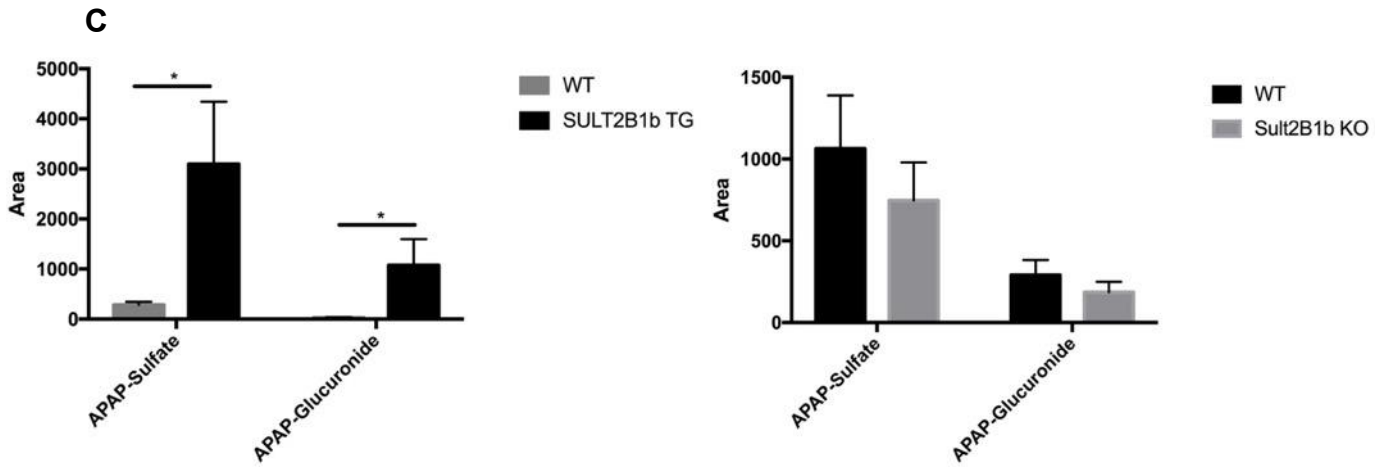


Figure 5. Profile of APAP metabolism in SULT2B1b TG and KO mice.
A Relative mRNA levels of *Ugt1*, *Gstα*, *Gstμ*, *Gstπ*, *Sult2a1* and *Cyp2e1* in livers from WT, SULT2B1b TG and KO mice treated with APAP. **B** Total GSH content in livers from WT, SULT2B1b TG and KO mice treated with APAP (n=3 per group). **C** LC-MS analysis of the content of APAP-Sulfate and APAP-Glucuronide in livers from WT, SULT2B1b TG and KO mice at 24 hours after APAP treatment. Data are expressed as mean ± SEM. **P* < 0.05; ***P* < 0.01.

4.0 DISCUSSION

Identification of the molecular mechanisms during pathogenesis of APAP-induced liver injury will provide promising therapeutic avenues for the treatment for APAP overdose. In this study, we first demonstrated that the hepatic overexpression of SULT2B1b enhanced APAP-induced acute liver injury in mice while ablation of Sult2B1b in mice conferred resistance to this liver injury. Furthermore, TG mice also showed increased hepatocyte death and inflammation, whereas KO mice reduced. Our previous study showed that SULT2B1b is transcriptionally regulated by HNF4α[35]. Thereby, we also identified that overexpression of Hnf4α to upregulate Sult2B1b sensitized WT mice to APAP hepatotoxicity. In contrast, this sensitization of HNF4α was abolished in Sult2B1b KO mice. Therefore, Sult2B1b is essential for regulation of HNF4α to APAP-induced liver injury.

SULT2B1b is a hydroxysteriod sulfotransferase which plays an important role in lipid and glucose metabolism, inflammatory responses and cell proliferation[52,53]. It has been reported that SULT2B1b is involved in a series of liver diseases, including metabolic syndrome[35], HCC[37], NASH and NAFLD[54]. Noted that SULT2B1b suppresses LXR activity[38,55]. We previously demonstrated that activation of LXR accelerates APAP clearance to prevent APAP toxicity[49]. Based on these, we are curious about the role of SULT2B1b itself in APAP hepatotoxicity and we propose upregulation of SULT2B1b sensitizes mice to APAP-induced liver injury. We followed our previous protocol to treat mice with 200mg/kg APAP by gavage after 16-hour fasting[49]. Because fasting accelerates GSH depletion and induces CYP2E1 expression to promote APAP toxicity[19]. As a result, consistent with our hypothesis, TG mice showed significant severe necrosis and increased serum ALT and AST levels, whereas KO mice alleviated APAP-induced liver injury

and reduced ALT and AST levels. In APAP-induced liver injury, it is believed that overdose of APAP is metabolized by CYPs to reactive intermediate NAPQI which depletes GSH, binds to cellular proteins and induce nuclear DNA fragmentation, leading to necrotic hepatocyte death[13]. So in this study, we assessed nuclear DNA fragmentation by TUNEL staining. On the other hand, hepatocyte necrosis eventually induces liver regeneration[56]. Liver regeneration is also known to be important for survival after APAP overdose[57]. Usually, more necrosis is followed by more cell proliferation. Furthermore, enhanced liver injury in SULT2B1b TG mice was also accompanied by an exacerbation of inflammation[58], as evidenced by increased hepatic expression of *Il-6* or *Il-1β*. In contrast, Il-1β decreased in Sult2B1b KO mice.

HNF4α is a key regulator of both hepatocyte differentiations during embryonic development and maintenance of a differentiated phenotype in adult[59]. It has been shown that HNF4α is essential for liver development, lipid and glucose metabolism, NASH, ALF and HCC, etc[46,48,60,61]. Therefore, the regulation of HNF4α and changes in its downstream are essential for liver development and function. To overexpress Hnf4α, we tried hydrodynamic transfection and adenovirus infection expressing Hnf4α in vivo respectively. By hydrodynamic transfection, hepatic expressions of Hnf4α and Sult2B1b rapidly increased at both mRNA and protein levels (data not shown). However, hydrodynamic transfection also induce severe liver injury with extravasated blood[62]. We found it is difficult to tell the damage caused by APAP or hydrodynamic. And APAP-induced liver damage was compensated probably because of liver regeneration. It is probably because of liver regeneration. Therefore, we overexpressed Hnf4α by adenovirus to upregulate Sult2B1b. But it takes a week to fully upregulate Sult2B1b so only Hnf4α protein increase was detected. Surprisingly, Hnf4α sensitized WT mice to APAP hepatotoxicity. To our knowledge, it is the first demonstration proved that HNF4α aggravated APAP-induced liver injury

via SULT2B1b upregulation. The mechanism will be illuminated. So SULT2B1b may have a directly or indirectly effect on APAP hepatotoxicity. Because adenovirus infection also induced mild liver damage leading to liver regeneration, the necrosis after APAP treatment in these mice was not as dramatic as simple APAP-treated mice. Then we were wondering whether the sensitization of HNF4α is dependent on SULT2B1b. As expected, Sult2B1b KO mice injected with Ad-Hnf4α showed similar liver injury to those injected with Ad-ctrl. Thereby, we concluded that HNF4α promoted APAP-induced liver injury via SULT2B1b upregulation.

Our results suggested that most of gene expressions of APAP metabolizing enzymes, except for Ugt1 in TG mice, did not change. It indicated that SULT2B1b may be dispensable for the metabolism of APAP. But for the total content of GSH, there was an abnormal reduce in TG mice. One possible reason is that dramatic necrosis results in GSH degradation. Because GSH depletion reflects the amount of excessive NAPQI, another possible explanation is that overexpression of hepatic SULT2B1b affected initial depletion of GSH levels. Further study will be needed to elucidate this issue. We preliminarily measured APAP metabolites in serum at 24 hours post-APAP. To our surprise, two nontoxic metabolites, APAP-sulfate and APAP-glucuronide, remarkably increased in SULT2B1b TG mice though no significant decrease in KO mice. We still need to analyze the concentration of APAP-cysteine in liver to further conclude whether SULT2B1b influences APAP metabolism.

In summary, we have uncovered a novel function of SULT2B1b in APAP-induced acute liver injury. Because HNF4α plays a pivotal role in liver development and function, the induction of SULT2B1b by HNF4α contributes to an important regulation to APAP hepatotoxicity. Thus, our results suggest that hepatic SULT2B1b may be a potential therapeutic target for APAP-induced liver injury.

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