




The Wnt/ β -catenin signaling pathway plays a role in drug-induced liver injury by regulating cytochrome P450 2E1 expression

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Abstract

Drug-induced liver injury (DILI) is a major cause of acute liver failure and drug withdrawal. Cytochrome P450 (CYP) 2E1 is involved in the metabolism of several drugs, and can induce liver injury through the production of toxic metabolites and the generation of reactive oxygen species. This study aimed to elucidate the role of Wnt/ β -catenin signaling in CYP2E1 regulation for drug-induced hepatotoxicity. To achieve this, mice were administered cisplatin or acetaminophen (APAP) 1 h after treatment with the CYP2E1 inhibitor dimethyl sulfoxide (DMSO), and histopathological and serum biochemical analyses were performed. APAP treatment induced hepatotoxicity, as evidenced by an increase in liver weight and serum ALT levels. Moreover, histological analysis indicated severe injury, including apoptosis, in the liver tissue of APAP-treated mice, which was confirmed by TUNEL assay. Additionally, APAP treatment suppressed the antioxidant capacity of the mice and increased the expression of the DNA damage markers γ -H2AX and p53. However, these effects of APAP on hepatotoxicity were significantly attenuated by DMSO treatment. Furthermore, the activation of Wnt/ β -catenin signaling using the Wnt agonist CHIR99021 (CHIR) increased CYP2E1 expression in rat liver epithelial cells (WB-F344), whereas treatment with the Wnt/ β -catenin antagonist IWP-2 inhibited nuclear β -catenin and CYP2E1 expression. Interestingly, APAP-induced cytotoxicity in WB-F344 cells was exacerbated by CHIR treatment and suppressed by IWP-2 treatment. Overall, these results showed that the Wnt/ β -catenin signaling is involved in DILI through the upregulation of CYP2E1 expression by directly binding the transcription factor β -cat/TCF to the *Cyp2e1* promoter, thus exacerbating DILI.

Keywords Cytochrome P450 2E1 · Wnt/ β -catenin signaling · Hepatotoxicity · Acetaminophen · Cisplatin

Introduction

Drug-induced liver injury (DILI) is a severe condition caused by various medications, including herbal drugs, xenobiotics, and dietary supplements, that induce abnormalities in liver function [1]. For instance, the representative first-line anti-tubercular treatments of isoniazid and rifampicin can cause hepatotoxicity [2, 3]. Isoniazid is metabolized to acetylhydrazine and hydrazine, which are further metabolized to toxic products by cytochrome P450 (CYP) [4]. Although the metabolites of rifampicin are non-toxic, they can induce isoniazid hydrolysis, which increases hydrazine production, especially in slow acetylators, thereby increasing isoniazid-induced toxicity [5]. As a first-generation anticancer drug used to treat various cancers, such as ovarian, testicular, and head and neck cancers [6, 7], the clinical use of cisplatin is frequently restricted by its various adverse effects, such as ototoxicity, nephrotoxicity, and hepatotoxicity [8]. Cisplatin

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is hydrolyzed to an active form that reacts with DNA [9], inhibiting DNA replication and transcription [9]. In addition to the inhibition of DNA synthesis, mitochondrial reactive oxygen species (ROS) production is known to be involved in cisplatin-induced apoptosis and liver necrosis [10, 11]. Acetaminophen (APAP), a leading cause of DILI, is commonly used for therapeutic purposes, including antipyretics and analgesics. Studies have shown that APAP overdose can cause acute liver failure (ALF) [12, 13] through hepatocyte cell death induced by mitochondrial dysfunction, oxidative stress, and DNA damage resulting from the activities of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) and cellular proteins [14]. Additionally, the inflammatory process and ROS produced by infiltrating neutrophils and macrophages contribute to hepatotoxicity [2, 15]. DILI is thought to occur via various pathological mechanisms.

CYP is a superfamily of hemoproteins that mediate the oxidative metabolism of several exogenous and endogenous compounds [16]. Among CYPs, CYP1A2 plays an important role in the metabolism of various compounds, including the activation of carcinogenic aryl compounds, such as benzo[a]pyrene [17]. As the most abundant CYP enzyme in the liver, CYP3A4 is known to catalyze approximately 50% of the metabolism of therapeutic agents, including the anticancer drug vinorelbine [18]. Additionally, lapatinib, an approved treatment for human epidermal growth factor receptor 2-positive breast cancers, is metabolized by CYP3A4 and CYP3A5, and chemically reactive metabolites produced by CYPs can induce hepatotoxicity [19]. Moreover, CYP2E1 is highly expressed in the liver and plays an important role in the metabolism of several drugs [20]. For instance, APAP-induced hepatotoxicity is caused by NAPQI, a toxic metabolite formed primarily by the CYP2E1 metabolic pathway [21]. Moreover, increased CYP2E1 expression has been observed in cells affected by cisplatin-induced hepatotoxicity [20]. *Cyp2e1*-knockout mice were resistant to toxicity caused by the metabolism of chemicals, such as 4-aminobiphenyl, carbon tetrachloride, and APAP. High-fat-diet-induced obesity was also alleviated in *Cyp2e1*-knockout mice [22–24]. Overall, these findings suggest that CYP2E1 is involved in the metabolism of various chemicals and drugs, and DILI.

Presently, NSAIDs and anti-infective, antineoplastic, cardiovascular, neurological, and gastrointestinal agents account for 10, 45, 13, 9, 6, and 4% of liver injuries, respectively [25]. Particularly, APAP toxicity accounts for 46% of all ALF in the USA and 40–70% in the UK and Europe [26]. Despite the important clinical impact of DILI, currently available information is insufficient. Considering that some of the medication-induced harmful effects could be attributed to the production of toxic metabolites during drug

metabolism [27], a study on CYP2E1, which is known to be a crucial factor in drug metabolism, free radical production, and subsequent oxidative stress [28], can be very helpful in reducing DILI. Many lines of evidence support that Wnt/ β -catenin signaling play an important role in CYP2E1 expression as a transcriptional regulator in vitro and in vivo [29, 30] as well as liver development and regeneration [31–33]. Therefore, the aim of this study was to elucidate the mechanisms by which CYP2E1 contributes to the development and progression of liver injury and, in particular, the role of the Wnt/ β -catenin signaling pathway during this process.

Materials and methods

Animal experimental procedures

Eight-week-old male ICR mice (Orient Bio, Seongnam, Korea) were housed in an environmentally-controlled room (22 ± 2 °C, 50–60% humidity, and 12 h light cycle) and supplied normal rat chow (Harlan 2918 C; Raon Bio, Yongin, Korea) and tap water *ad libitum*. In Experiment 1, the mice were randomly assigned into three groups: mice in group I were treated with a single intraperitoneal injection of normal saline and orally treated with vehicle (distilled water) 1 h later; mice in group II were intraperitoneally administered normal saline once and orally administered a single dose of 300 mg/kg APAP (Sigma, St. Louis, MO, USA) 1 h later; and mice in group III were intraperitoneally administered dimethyl sulfoxide (DMSO, 20% v/v in normal saline; Sigma) at 10 μ L per gram body weight and orally administered a single dose of 300 mg/kg APAP 1 h later. The mice were euthanized 24 h after vehicle or APAP treatment. In Experiment 2, the mice were divided into six groups: Mice in group I were treated with a single intraperitoneal injection of vehicle (normal saline); mice in group II were intraperitoneally administered DMSO (20% v/v in normal saline) at 10 μ L per gram body weight; mice in groups III and IV were treated with a single intraperitoneal injection of 25 mg/kg of cisplatin (Sigma) with or without DMSO pretreatment 1 h before cisplatin exposure; and mice in groups V and VI were injected with a single intraperitoneal dose of 40 mg/kg of cisplatin with or without DMSO pretreatment 1 h before cisplatin exposure. The mice were euthanized 24 h after vehicle or cisplatin treatment. The dose, duration, and administration route of APAP were adopted from previous study [34]. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Catholic University of Korea (CUK-IACUC-2017-014-06, 2019-029).

Serum biochemical analysis

Blood samples were obtained using a BD Vacutainer™ SST™ II Advance (BD Diagnostic, Sparks, MD, USA) and then centrifuged at 3,000 rpm for 15 min at 4 °C. After centrifugation, serum was collected and stored at -80 °C before analysis. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using an automatic analyzer (BS-220, Mindray, China; ADVIA 2120i, SIEMENS, Germany).

Histology and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Formalin fixed (10%) liver tissues were paraffinized, cut into 4 µm sections using rotary microtome (Leica RM2245, Leica, Wetzlar, Germany) for hematoxylin and eosin (H&E) staining, and the stained sections were viewed using a light microscope (Leica, Hamburg, Germany). Additionally, TUNEL assay was performed on paraffin-embedded mouse liver tissue sections using an apoptosis detection kit (206386; Abcam, Cambridge, MA, USA), according to the manufacturer's instructions. Briefly, paraffin-embedded tissues were deparaffinized in xylene and rehydrated using a graded series of ethanol. After washing with Tris-buffered saline (TBS) buffer (0.150 M NaCl and 20 mM Tris-HCl, PH 7.6), the slides were permeabilized with a proteinase K solution and equilibrated with TdT equilibration buffer. Thereafter, the tissue slides were labeled with a TdT labeling reaction mixture for 90 min at room temperature and processed sequentially to terminate the labeling, blocking, and conjugation. Finally, the slides were treated with 3, 3'-diaminobenzidine solution for 20 min, washed with tap water, counterstained with methyl green solution, and images were acquired using a light microscope (Leica).

Western blot

Frozen mouse liver tissue and cells were lysed using RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing Halt™ Protease Inhibitor single-use cocktail EDTAfree (Thermo Fisher Scientific, Waltham, MA, USA). The lysates were centrifuged at 15,000 rpm for 30 min at 4 °C to collect supernatants, and protein concentrations were estimated using Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA). Thereafter, the proteins were subjected to SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% skim milk in phosphate-buffered saline with 0.1% Tween® 20 detergent (PBST) solution at room temperature for 1 h, followed by incubation

with primary antibodies (Supplementary Table 1) overnight at 4 °C. Subsequently, the membranes were washed three times with PBST, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 h at room temperature. The protein signals were developed using a Clarity™ Western ECL substrate (Bio-Rad), and protein expression was visualized using the ChemiDoc™ XRS + System (Bio-Rad).

RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, San Diego, CA, USA), and RNA quality and quantity ($A_{260}/A_{280} > 1.8$ was considered qualified) were determined using Nanodrop ND-2000 (Thermo Fisher Scientific). Thereafter, 1 µg of RNA was transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). For reverse transcription real-time PCR, cDNA was mixed with THUNDERBIRD SYBR qPCR Mix (Toyobo) and amplified using a CFX Connect Real-Time PCR detection system (Bio-Rad). The Cq values of all PCR reactions were obtained using the CFX Manager™ software (Bio-Rad). The relative expression of target genes was normalized to that of *GAPDH* (internal control) and calculated using the $2^{-\Delta\Delta Cq}$ method. The primer sequences used are listed in Supplementary Table 2.

Cell culture and viability assessment

WB-F344 cells were obtained from the Seoul National University Hospital (Seoul, Korea). The cells were cultured in Dulbecco's Modified Eagle's Medium F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (WelGENE, Daegu, Korea) and 1% antibiotic-antimycotic (Gibco) at 37 °C in a 5% CO₂ incubator. Cells were cultured in 60-mm dish (0.6×10^6 cells) or 100-mm dish (1.5×10^6 cells) for 24 h and treated with 2–5 µM of CHIR99021 (CHIR, StemCell Technologies, Vancouver, Canada) for 24 h or 5–20 µM of IWP-2 (Sigma) for 48 h, and cell viability was detected using the CCK-8 kit (Dojindo, Tokyo, Japan). Briefly, the cells were seeded into a 96-well plate at a density of 5×10^3 cells/well for 24 h, treated with APAP in the presence or absence of CHIR or IWP-2, followed by the addition of CCK-8 reagent solution to each well and incubated for 2 h at 37 °C. Thereafter, the absorbance was measured at 450 nm using SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA).

Luciferase assay

To determine the promoter region responsible for *Cyp2e1* gene expression, fragments of different lengths of the 5'-promoter region of mouse *Cyp2e1* were amplified by PCR using different pairs of primers (Supplementary Table 2). These primers were then flanked by the restriction enzyme cloning site, KpnI site at the 5'-end, and Bgl II site at the 3'-end of the amplified fragments. PCR was performed using Top DNA Polymerase (BioNeer, Daejeon, Korea). The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), digested with KpnI and BglII, and cloned into a pGL3-basic vector (Promega). The digested products were ligated using T4 DNA Ligase (Takara, Tokyo, Japan). Competent *E. coli* DH5 α cells were treated with MgCl₂ and CaCl₂, and recombinant pGL3 vectors were transformed into *E. coli* DH5 α cells using the heat shock method. To achieve this, the pGL3 vectors were added to the competent cells and the mixture was placed on ice for 30 min, exposed to heat shock at 42 °C for 90 s in a water bath, followed by cooling on ice for 2 min, and the addition of 900 μ L of LB broth. After incubation for 1 h at 37 °C and 200 rpm, 100 μ L of the cells were spread on an LB agar plate containing ampicillin. Each colony was then cultured in LB broth and the plasmid was isolated using the Qiagen Miniprep kit (Qiagen, Valencia, CA, USA). Transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific), and the cells were cultured in 48-well plates. After treatment with IWP-2 or CHIR, cells were washed, lysed with lysis buffer, and luciferase activity was measured using GloMax Explorer dual-luciferase reporter assay kit (Promega), according to the manufacturer's instructions, with firefly and Renilla luciferase as the experimental promoter and control reporters, respectively. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

Analysis of putative factor binding site of β -catenin in *Cyp2e1* promoter

The 1.0 kb region flanking the 5' end of the mouse *Cyp2e1* gene was retrieved from the NIH/NCBI Entrez gene database (<http://www.ncbi.nlm.nih.gov/gene>), and putative transcription factor-binding sites on the *Cyp2e1* promoter region were determined using TRANSFAC PATCH™ 1.0 program (<http://gene-regulation.com/index2.html>).

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed using the Imprint® Chromatin Immunoprecipitation Kit (Sigma), according to the manufacturer's instructions. Briefly, WB-F344 cells were treated

with 20 μ M of IWP-2 for 48 h, cross-linked with 1% formaldehyde for 10 min, and neutralized with 1.25 M of glycine at room temperature for 5 min. After washing with ice-cold PBS, cross-linked cells were lysed by vortexing in a nuclei preparation buffer. The pelleted nuclei were sheared into small fragments by sonicating in a shearing buffer supplemented with protease inhibitors (200 μ L/10 million WB-F344 cells). The sheared DNA was then centrifuged, and aliquots were analyzed by agarose gel electrophoresis to verify DNA fragment size (200–1000 bp). Before immunoprecipitation, input controls were sampled and stored in vials. Immunoprecipitation was performed using an anti-monoclonal mouse β -catenin antibody (BD Biosciences), with normal mouse IgG used as the negative control. Unbound DNA was removed using an IP wash buffer and bound DNA was extracted by cross-link reversal using a DNA release buffer containing proteinase K in the input vials and each sample. After the collected DNA was purified using GenElute binding column G, it was amplified using the designed PCR primers (Supplementary Table 2).

Statistical analysis

Data were presented as mean \pm standard deviation (SD). Significant differences between two or more groups was determined using Student's *t*-test or one-way analysis of variance (ANOVA), and mean values were considered significant at $p < 0.05$ or $p < 0.01$.

Results

Effect of CYP2E1 inhibitor in cisplatin- and APAP-induced hepatotoxicity

To investigate the effects of CYP2E1 on cisplatin- and APAP-induced hepatotoxicity, mice were administered DMSO, a CYP2E1 inhibitor [35, 36], after cisplatin- or APAP treatment. Although treatment with 25 and 40 mg/kg of cisplatin did not induce any significant changes in the absolute and relative weights of liver tissues (Supplementary Fig. 1a), cisplatin-treated mice had significantly higher ALT and AST levels compared with the control group (Supplementary Fig. 1b). Importantly, DMSO did not affect cisplatin-induced hepatotoxicity. In contrast, mice administered 300 mg/kg of APAP had significantly higher absolute and relative liver weights compared with untreated mice. However, pretreatment with DMSO (20% v/v in normal saline) significantly attenuated the APAP-induced increase in liver weight (Fig. 1a). Also, serum ALT level was markedly increased in APAP-treated mice than the control mice ($p = 0.051$) whereas DMSO pretreatment suppressed

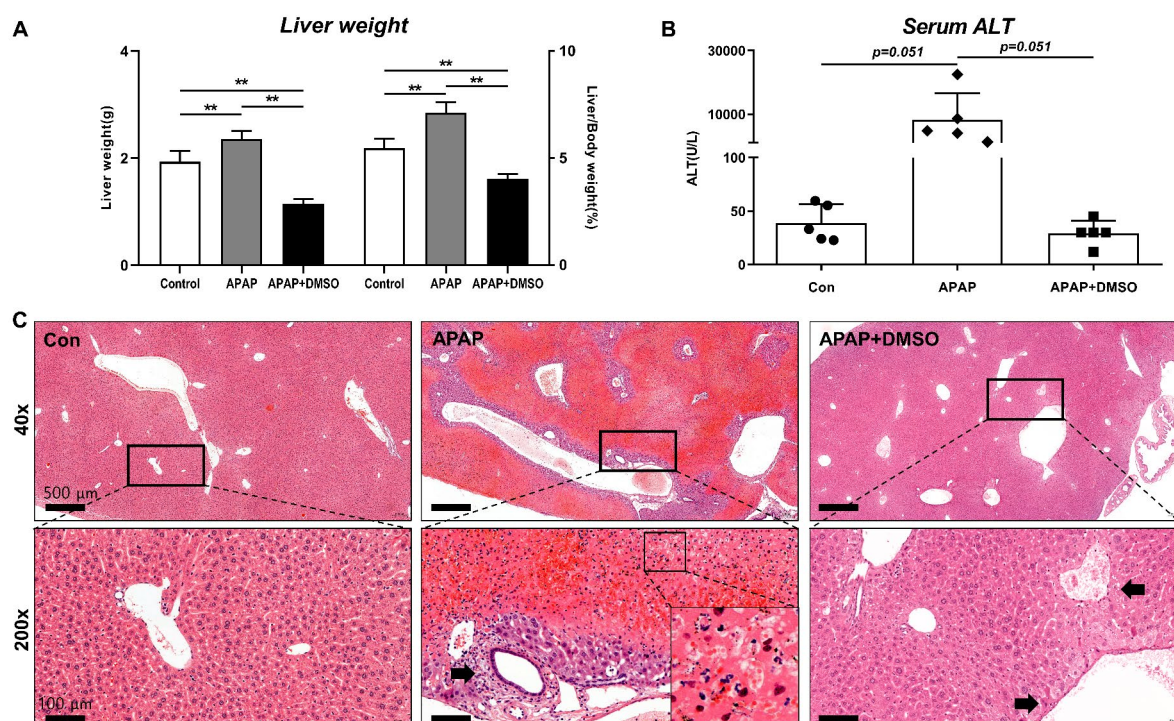


Fig. 1 Effect of CYP2E1 inhibitor DMSO on APAP-induced hepatotoxicity in mice. (A, B) Effects of CYP2E1 inhibitor DMSO on absolute and relative liver weights (A) and serum alanine aminotransferase (ALT) levels (B) in APAP-induced hepatotoxicity. (C) Representative

H&E staining of the liver of APAP-treated mice show necrosis (inserted box) and inflammation (arrow). Data are expressed as means \pm standard deviation (SD); ** $p < 0.01$. Con, control; DMSO, dimethyl sulfoxide; APAP, acetaminophen; H&E, hematoxylin and eosin

APAP-induced ALT increase, which is similar to control groups ($p = 0.051$) (Fig. 1b).

H&E staining indicated that in the liver tissue of APAP-treated mice, there was extensive necrosis of the central lobule to the middle and peripheral regions of the liver lobules, with hemorrhages and fibrin. In the affected area, many liver cells were lost, shrunk, or hypereosinophilic with pyknotic nuclei. The portal connective tissue was slightly dilated and infiltrated with inflammatory cells consisting of lymphocytes, macrophages, and fewer neutrophils. In contrast, there was an obvious improvement in the liver morphology of DMSO pre-treated mice (Fig. 1c). Multifocally, hepatocytes at the margin of periportal and centrilobular areas were minimally to mildly swollen with pale eosinophilic, occasionally microvacuolated cytoplasm.

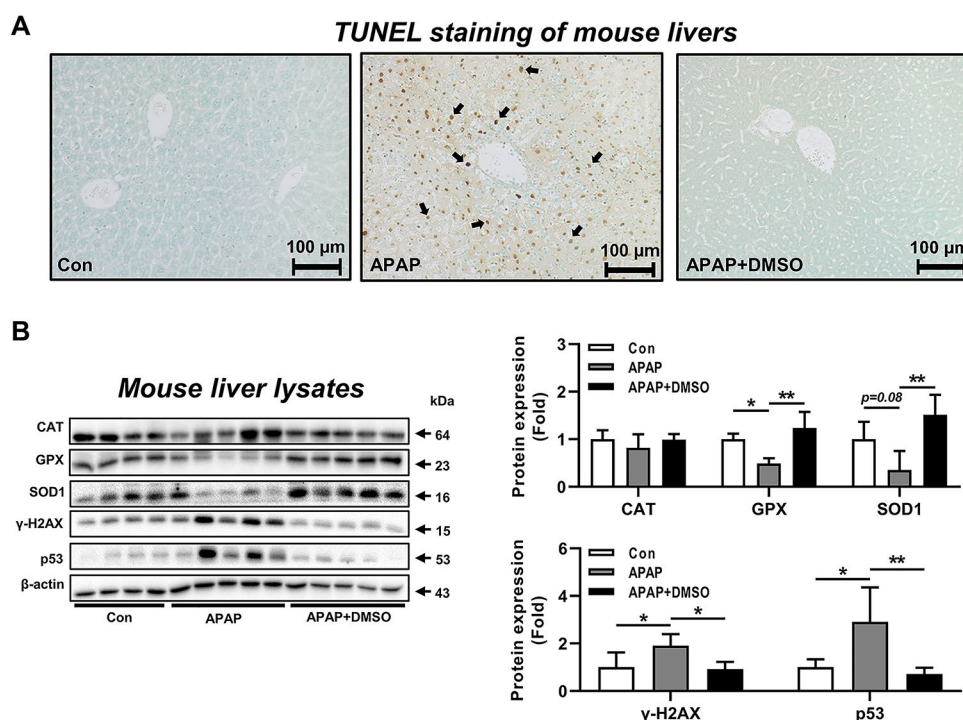
Additionally, several TUNEL-positive cells were detected in the liver sections of APAP-treated mice, indicating apoptosis, whereas TUNEL-positive cells were rarely detected in the liver sections of untreated and DMSO pre-treated mice (Fig. 2a). Moreover, APAP-treated mice had lower expression of antioxidant enzyme glutathione peroxidase (GPX) and superoxide dismutase 1 (SOD1), compared with untreated mice. However, co-treatment with DMSO significantly increased the levels of these antioxidant enzymes compared with those of APAP-treated mice

(Fig. 2b). Furthermore, the protein levels of γ -H2AX and p53, DNA damage markers, were examined to determine whether APAP-induced cell injury-involved DNA damage [37, 38]. APAP treatment significantly increased γ -H2AX and p53 expression levels, whereas DMSO pre-treated mice showed similar expression levels to those of untreated mice. Overall, these results suggest that inhibiting CYP2E1 expression could prevent APAP-induced DNA damage and oxidative stress.

Involvement of Wnt/ β -catenin signaling in the regulation of CYP2E1 expression

In the present study, WB-F344 cells were treated the Wnt agonist CHIR or the Wnt antagonist IWP-2 to elucidate the role of the Wnt/ β -catenin signaling in CYP2E1 regulation [39]. Treatment with 3 μ M of CHIR for 24 h promoted β -catenin nuclear translocation in the cells, as well as CYP2E1 expression at the protein and mRNA levels compared with untreated cells (Fig. 3a and c). In contrast, treatment with 20 μ M of IWP-2 for 48 h caused a decrease in nuclear β -catenin levels in WB-F344 cells (Fig. 3d), and downregulated both the protein and mRNA levels of CYP2E1 (Fig. 3e and f).

Fig. 2 Effect of CYP2E1 inhibitor DMSO on APAP-induced apoptosis, oxidative stress, and DNA damage in mice. (A) Representative liver histological sections were stained with TUNEL (Bar = 100 μ m). Black arrows show TUNEL-positive cells. (B) Immunoblot analysis for antioxidant enzymes (catalase, GPX, and SOD1) and DNA damage markers (γ -H2AX and p53) in whole liver lysates. β -actin was used as loading control for protein expression analysis. Data were expressed as means \pm standard deviation (SD); * p < 0.05 and ** p < 0.01. Con, control; DMSO, dimethyl sulfoxide; APAP, acetaminophen



To further explore the role of Wnt/ β -catenin signaling in the activation of *Cyp2e1* promoter, we identified four putative binding sites of β -catenin (-605/-601, -440/-436, -405/-400, and -153/-149) in the promoter region 1.0 kb upstream of the transcription start site of the mouse *Cyp2e1* gene using the TRANSFAC PATCHTM 1.0 program. There was an increase in the luciferase activity of CHIR-treated WB-F344 cells transfected with the *Cyp2e1* promoter construct (from -482 to -49 bp) containing three β -catenin/TCF4 binding sites (-440/-436, -405/-400, and -153/-149) (Fig. 3g) compared with the control group (Fig. 3h). In contrast, the inhibition of Wnt/ β -catenin signaling by IWP-2 significantly reduced luciferase activity.

ChIP assay was performed to elucidate the role of Wnt/ β -catenin signaling in the regulation of *Cyp2e1* promoter activation. WB-F344 cells were treated with 20 μ M of IWP-2 for 48 h. DNA purified from the immunoprecipitated DNA-protein complex was subjected to PCR amplification using oligonucleotide primers flanking four putative β -catenin/TCF4 binding sites identified in the *Cyp2e1* promoter regulatory region (Fig. 3i and j). The ChIP analysis revealed that β -catenin binds directly to TCF4 binding site 1 in *Cyp2e1* promoter region (Fig. 3k). Particularly, treatment with 20 μ M of IWP-2 for 48 h markedly reduced the binding of β -catenin to TCF4 binding site 1 in the promoter region of *Cyp2e1*, indicating that *Cyp2e1* promoter activation could be partially associated with the binding of β -catenin.

Effects of Wnt/ β -catenin signaling in APAP-induced cytotoxicity

To confirm the effects of Wnt/ β -catenin signaling on APAP-induced cytotoxicity, cells were treated with APAP in the absence or presence of CHIR and IWP-2. APAP treatment reduced cell viability in a dose-dependent manner; however, co-treatment with 3 μ M of CHIR for 24 h exacerbated APAP-induced cytotoxicity (Fig. 3l). In contrast, IWP-2-mediated inhibition of Wnt/ β -catenin signaling (20 μ M of IWP-2) attenuated APAP-induced cytotoxicity compared with that in cells treated with APAP only (Fig. 3m). These results suggest that CYP2E1 regulate APAP-mediated cytotoxicity via the Wnt/ β -catenin signaling pathway.

Discussion

CYP are a superfamily of hemoproteins that mediate the oxidative metabolism of several exogenous and endogenous compounds [16]. CYP2E1 is primarily responsible for the metabolism of chemicals and drugs, such as ethanol, acetone, organic solvents, chlorzoxazone, and APAP [28]. APAP has been shown to induce hepatotoxicity through CYP2E1-mediated production of the highly reactive metabolite NAPQI [21, 40]. Although NAPQI can be detoxified by conjugation to glutathione (GSH) and excreted under normal conditions, when hepatic GSH is exhausted by APAP overdose, NAPQI covalently binds to various cellular proteins, leading to

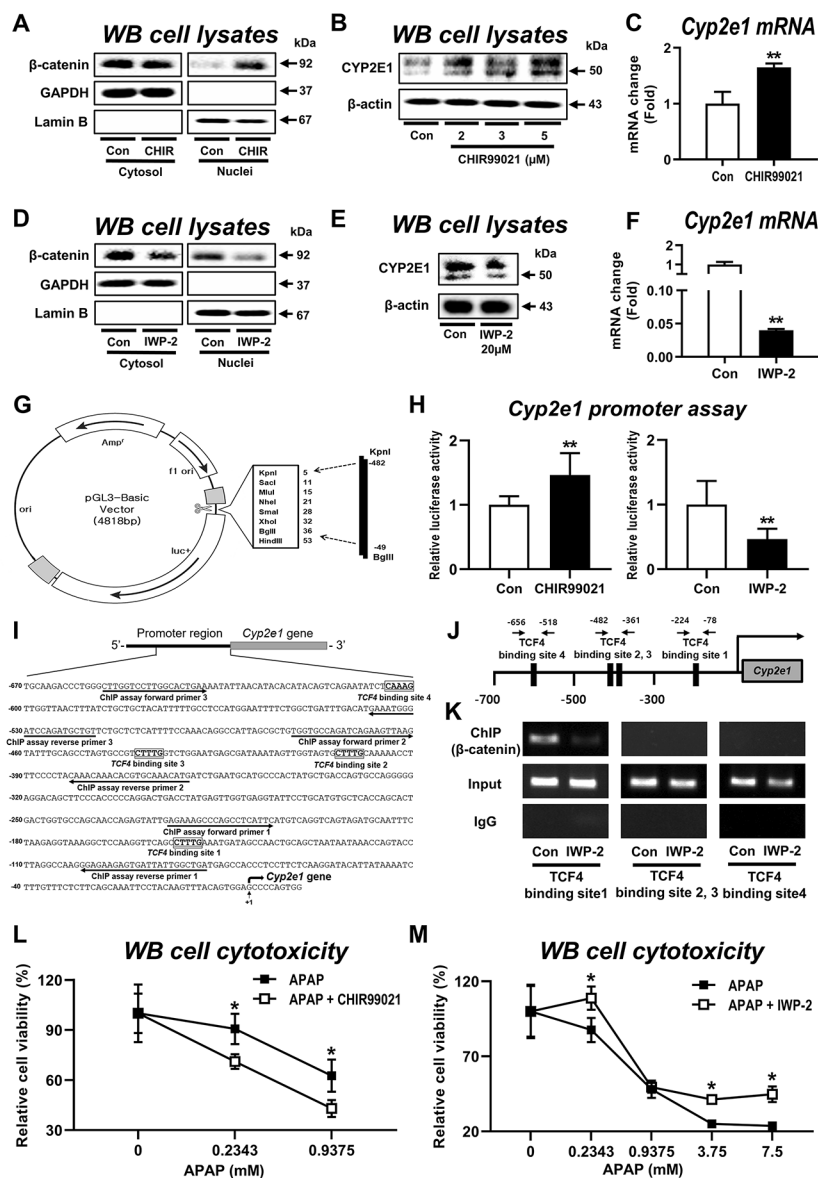


Fig. 3 Role of Wnt/β-catenin signaling in CYP2E1 expression in WB-F344 cells. (A) Immunoblot analysis for β-catenin in cytosolic and nuclear fractions of protein lysates isolated from WB-F344 cells treated with 3 μM of CHIR for 24 h. (B) Immunoblot analysis for CYP2E1 in whole lysates isolated from WB-F344 cells treated with 2, 3, and 5 μM of CHIR for 24 h. (C) Relative mRNA levels of *Cyp2e1* in WB-F344 cells treated with 3 μM of CHIR for 24 h. (D) Immunoblot analysis for β-catenin in cytosolic and nuclear fractions of protein lysates isolated from WB-F344 cells treated with 20 μM of IWP-2 for 48 h. (E) Immunoblot analysis for CYP2E1 in whole cell lysates isolated from WB-F344 cells treated with 20 μM of IWP-2 for 48 h. (F) Relative mRNA levels of *Cyp2e1* in WB-F344 cells treated with 20 μM of IWP-2 for 48 h. (G) Construction of the luciferase reporter plasmid with the *Cyp2e1* promoter (from -482 to -49 bp) containing TCF4, the β-catenin binding sites (-440/-436, -405/-400, and -153/-149). (H) *Cyp2e1* promoter activity in WB-F344 cells transfected with a pGL3 vector containing *Cyp2e1* promoter, followed by treatment with 3 μM of CHIR for 24 h or 20 μM of IWP-2 for 48 h. (I) Sequence of the *Cyp2e1* promoter region. Boxed nucleotides are

putative β-catenin binding sites, TCF4. Primer sequences are indicated by arrows with right (forward primer) and left (reverse primer) directions. (J) ChIP assay to identify β-catenin binding site on TCF4 in the *Cyp2e1* promoter region. The panel shows the schematic overview of the TCF4 sites in the *Cyp2e1* promoter (the black box represents the TCF4 binding sites for each primer sets). (K) WB-F344 cell extracts were subjected to ChIP assay using antibodies specific for β-catenin, nonimmune IgG, and input. Precipitated DNA was amplified by real-time qPCR with primers flanking the predicted TCF4 binding sites of the *Cyp2e1* promoter. The panel is a representative DNA gel image showing the binding of β-catenin protein to different TCF sites in the *Cyp2e1* promoter. (L, M) Effects of CHIR 3 μM for 24 h (L) and IWP-2 20 μM for 48 h (M) in APAP-induced cytotoxicity in WB-F344 cells. GAPDH and Lamin B were used as loading controls for cytosolic and nuclear proteins, respectively. GAPDH and β-actin were used as loading controls for mRNA and protein expression analysis, respectively. Data are expressed as means ± SD (* p < 0.05 and ** p < 0.01). Con, control; APAP, acetaminophen; CYP2E1, cytochrome P450 2E1; CHIR, CHIR99021; TCF, T-cell factor

mitochondrial dysfunction and oxidative stress-induced hepatotoxicity [21]. In the present study, APAP induced severe hepatotoxicity, as evidenced by an increase in liver weight and serum ALT levels (Fig. 1a and b), histological changes (Fig. 1c), and a decrease in the activities of antioxidant enzymes (Fig. 2b). Additionally, APAP treatment induced apoptosis (Fig. 2a) and DNA damage in hepatocytes (Fig. 2b), which are the major causes of hepatotoxicity [21, 37]. Previous studies have shown the *Cyp2e1* knock-down protected mice against toxicity induced by chemicals and drugs, such as lipopolysaccharide and APAP [41, 42]. Notably, pretreatment with the CYP2E1 inhibitor DMSO significantly prevented APAP-induced hepatocyte cell death and oxidative stress, indicating the critical role of CYP2E1 in APAP-induced hepatotoxicity. This result is in agreement with a previous study showing that DMSO treatment attenuated APAP-induced hepatotoxicity through the inhibition of CYP2E1 [43]. Interestingly, pretreatment with the CYP2E1 inhibitor DMSO [35, 36] did not affect cisplatin-induced hepatotoxicity (Supplementary Fig. 1a and 1b). Cisplatin enters the cell *via* passive diffusion and active transport through the copper membrane carrier copper transporter receptor 1, and is then hydrolyzed to an active form that cross-links adjacent guanine and adenine, which is crucial for cytotoxicity. As a result, cisplatin-DNA complexes interfere with DNA replication and induce cell cycle arrest and apoptosis [9]. Therefore, despite the broad roles of CYP2E1 in ROS generation and subsequent oxidative stress in the liver through interaction with cisplatin [20], the findings of the present study suggest that cisplatin hepatotoxicity may be through other major mechanisms other than CYP2E1-induced oxidative stress.

CYP2E1 interacts with several cellular signaling molecules and is regulated by transcription factors, such as NF- κ B, HNF1 α , and β -catenin [44–46]. NF- κ B regulates the gene expression of various hepatic CYP enzymes, including CYP2E1 [47]. Previous studies have identified several putative binding sites for NF- κ B in *Cyp2e1* promoter region [45]. Moreover, CYP2E1 expression is dependent on the Wnt/ β -catenin signaling pathway [48]. Wnt/ β -catenin signaling is activated through Wnt ligand binding to Frizzled, a cell membrane receptor, and the co-receptor lipoprotein receptor-related proteins 5 and 6 (LRP-5/6) [47, 49]. Binding of the Wnt ligand inactivates GSK3 β , inhibiting β -catenin phosphorylation, resulting in β -catenin accumulation and translocation to the nucleus. In the nucleus, β -catenin binds to TCF/LEF to activate the transcription of target genes. Based on the regulation of target genes, Wnt/ β -catenin signaling has been shown to play a major role in liver regeneration [50, 51]. Overexpression of Wnt/ β -catenin signaling in a small-for-size liver graft model has been reported to enhance liver proliferation and regeneration [52]. Wnt/ β -catenin signaling is a crucial pathway for hepatic development,

regeneration, and regulation of hepatic energy metabolism and mitochondrial function [31–33]. Overall, these findings suggest that Wnt/ β -catenin signaling plays an important role in liver regeneration following various liver injuries. Wnt/ β -catenin signaling not only contributes to the development of liver fibrosis but also elevates the activation and proliferation of hepatic stellate cells (HSCs) [53]. Several studies have indicated that the blockage or downregulation of Wnt/ β -catenin signaling contributes to the inhibition of HSCs activation and fibrogenesis [54, 55]. Notably, Wnt signaling, which affects liver pathology, may be related to CYP2E1 expression. A reduction in the expression of several CYP enzymes, particularly CYP2E1, has been observed in hepatocyte-specific β -catenin knockout mice [48]. Another study reported a decrease in CYP2E1 expression in the Wnt/ β -catenin signaling receptor LRP5/6 knockout mice [29]. Moreover, there was a significant increase in CYP2E1 mRNA and protein expression in mouse liver tumor cells with altered β -catenin expression [30]. However, studies on the effect of Wnt/ β -catenin signaling on DILI via the regulation of CYP2E1 expression are limited.

β -catenin translocates to the nucleus by activating Wnt/ β -catenin signaling and functions as a co-factor for the TCF/LEF family of transcription factors to regulate target gene expression. Along with axin-2, MYC, cyclin D1, and glutamine synthetase [56], CYP2E1 is known to be regulated by β -catenin [48]. Based on the prediction results of putative binding sites of β -catenin in the *Cyp2e1* proximal promoter region using TRANSFAC PATCHTM 1.0, a *Cyp2e1* promoter construct containing TCF binding sites (–440/–436, –405/–400, and –153/–149) was designed in the present study, and *Cyp2e1* transcription levels were regulated by Wnt/ β -catenin signaling (Fig. 3h). Importantly, it was observed that the translocation of β -catenin into the nucleus, which can be regulated by the Wnt agonist CHIR and Wnt antagonist IWP-2, was positively related to CYP2E1 expression at both the mRNA and protein levels (Fig. 3a and f). Moreover, ChIP assay indicated that IWP-2 stimulation caused inhibition of β -catenin to bind directly to the *Cyp2e1* promoter region (Fig. 3k). Interestingly, co-treatment with the Wnt agonist CHIR promoted APAP-induced decrease in WB-F344 cell viability (Fig. 3l), whereas the Wnt antagonist IWP-2 attenuated APAP-mediated cytotoxicity (Fig. 3m), indicating that the activation of the Wnt/ β -catenin signaling may be closely associated with the aggravation of cytotoxicity through the upregulation of CYP2E1 expression. β -catenin translocated into the nucleus binds to TCF4 binding sites in the *Cyp2e1* promoter region and induces *Cyp2e1* transcription, resulting in the induction of CYP2E1 expression to promote APAP-induced toxicity.

In conclusion, the results of the present study showed that Wnt/ β -catenin signaling plays a role in DILI by regulating CYP2E1-mediated APAP toxicity. The Wnt/ β -catenin signaling plays an important role not only in the proliferative response

of hepatocytes but also in the development of hepatic fibrosis and HSCs activation during liver injury and regeneration [53]. Despite these various effects of Wnt/ β -catenin signaling, our findings suggest that it affects the upregulation of *Cyp2e1* gene expression by direct interaction of β -catenin with a consensus binding site within *Cyp2e1* promoter region, thus elevating the levels of CYP2E1 mRNA and protein. These further lead to aggravate APAP-induced hepatotoxicity both in vitro and in vivo. Overall, CYP2E1 may be a potential therapeutic strategy to prevent acute liver toxicity in DILI.

Abbreviations

ALT	alanine aminotransferase
ALF	acute liver failure
APAP	acetaminophen
AST	aspartate aminotransferase
ChIP	chromatin immunoprecipitation
CHIR	CHIR99021
CYP	Cytochrome P450
DILI	Drug-Induced Liver Injury
DMSO	dimethyl sulfoxide
GSH	glutathione
GPX	glutathione peroxidase
H&E	hematoxylin and eosin
HSCs	hepatic stellate cells
LEF	lymphoid enhancer factor
LRP-5/6	lipoprotein receptor-related proteins 5 and 6
NAPQI	N-acetyl-p-benzoquinone imine
PBST	phosphate-buffered saline with 0.1% Tween ® 20 detergent Pt platinum
ROS	reactive oxygen species
SOD1	superoxide dismutase 1
TBS	tris-buffered saline
TCF	T-cell factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Author Contribution All authors contributed to the study conception and design. Material preparation and data collection were performed by YSS, DBH, DHW, SYK, and CK. Data analysis were performed by YSS, DBH, JWP, YJ, and JWY. The first draft of the manuscript was written by YSS and DBH. And, writing-review, editing and supervision were performed by JWY. All authors read and approved the final manuscript.

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Data Availability Data generated and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Declarations

Ethics approval All animal experimental procedures were performed after approval by the Institutional Animal Care and Use Committee at The Catholic University of Korea.

Conflict of Interest The authors declare that they have no competing financial interests with respect to this study.

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