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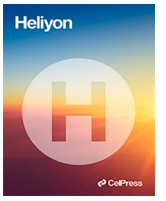

Research article

Blockade of 11β-hydroxysteroid dehydrogenase type 1 ameliorates metabolic dysfunction-associated steatotic liver disease and fibrosis

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ABSTRACT

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a key enzyme involved in the conversion of cortisone to active cortisol in the liver. Elevated cortisol levels can trigger oxidative stress, inflammation, and hepatocyte damage, highlighting the importance of 11β-HSD1 inhibition as a potential therapeutic approach. This study aimed to explore the effects of INU-101, an inhibitor of 11β-HSD1, on the development of metabolic dysfunction-associated steatotic liver disease (MASLD) and fibrosis. Our findings demonstrated that INU-101 effectively mitigated cortisol-induced lipid accumulation, reactive oxygen species generation, and hepatocyte apoptosis. Furthermore, 11β-HSD1 inhibition suppressed hepatic stellate cell activation by modulating β-catenin and phosphorylated SMAD2/3. INU-101 administration significantly reduced hepatic lipid accumulation and liver fibrosis in mice fed fast-food diet. This study suggests that INU-101 holds promise as a clinical candidate for treating MASLD and fibrosis, offering potential therapeutic benefits by targeting the intricate processes involving 11β-HSD1 and cortisol regulation in the liver.

1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by hepatic steatosis accompanied by pathological features such as inflammation, necrosis, and fibrosis [1]. MASLD is also associated with severe non-liver-related diseases, including cardiovascular diseases and malignancies [2]. Despite the increasing prevalence of MASLD, current therapeutic options remain limited and largely ineffective at addressing the complex metabolic dysfunctions underlying the disease [3]. Commonly used

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medications, such as antioxidant/hepatoprotective drugs like pioglitazone and obeticholic acid (OCA), provide symptomatic relief but fail to target the root causes of lipid dysregulation and fibrosis in MASLD [4]. Resmetirom is the only Food and Drug Administration approved drug specifically designated for metabolic dysfunction-associated steatohepatitis(MASH) with liver fibrosis [5], yet its scope of action is confined to specific pathways, leaving a substantial unmet need for broader and, more comprehensive therapeutic strategies. Therefore, the development of effective treatments for MASLD is crucial.

Glucocorticoids (GCs) are a class of steroid hormones predominantly synthesized and secreted by the zona fasciculata of the adrenal cortex; among them, cortisol is most prominent and functionally significant in humans [6]. These hormones regulate the biosynthesis and metabolism of glucose, lipids, and proteins. Moreover, they possess immunosuppressive, anti-inflammatory, antitoxic, and anti-nausea properties [7]. However, dysregulated GC activity, particularly excessive cortisol production, has been implicated in the exacerbation of metabolic disorders, including MASLD, by increasing lipid accumulation in metabolically active tissues [8]. Excess cortisol and GC levels can induce MASLD by increasing the accumulation of free fatty acids in the cytoplasm [9]. When the intracellular free fatty acids surpass anabolic and catabolic requirements, the excess lipids are converted to triglycerides and stored in lipid microdroplets, leading to lipotoxicity and accelerating the development of MASLD and fibrosis [10]. Therefore, controlling excess cortisol and GCs is important to regulating lipid metabolism and MASLD development. Furthermore, hypercortisolemia, a condition characterized by elevated cortisol levels, is a causal factor for MASLD [11]. For instance, hepatic steatosis was observed in 20 % of patients with Cushing's syndrome, a disorder caused by prolonged exposure to cortisol. This highlights the importance of exploring new therapeutic targets that can modulate GC activity, thereby offering a more effective approach to managing MASLD.

Recent studies have revealed a correlation between the enzyme activity of two isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD) and GC levels, particularly highlighting the involvement of 11β-HSD type 1 (11β-HSD1) in the pathogenesis of metabolic syndrome [12]. 11β-HSD1 is highly expressed in both rodent and human liver, with concentrations approximately tenfold higher than those in adipose tissues [13]. This enzyme belongs to the short-chain dehydrogenase/reductase protein superfamily and functions as an NAD(P)H-dependent microsomal enzyme [14]. In the presence of NADPH, it converts intracellular inactive cortisone into physiologically active cortisol [15]. The expression and/or activity of adipose tissue 11β-HSD1 in human obesity is closely related to the upregulation of GC receptors [16].

Given the critical role of 11β-HSD1 in the regulation of GC activity and its association with metabolic syndrome, targeting 11β-HSD1 represents a promising therapeutic approach for MASLD and liver fibrosis. In this study, we investigated the potential of 11β-HSD1 inhibition as a treatment for MASLD and liver fibrosis. Extensive studies on transgenic 11β-HSD1 knockout mice [17] and therapeutic inhibitors [18,19] have led to their progression to phase 2 trials [20–22]. However, there is currently a lack of comprehensive studies on the use of 11β-HSD1 inhibitors in mouse models of hepatic steatosis, creating a gap in our understanding of their potential therapeutic effects in MASLD. To address this, we used a fast-food diet (FFD) to induce MASLD in mice and evaluated the effects of 11β-HSD1 inhibitors in this model. This approach enabled us to explore the efficacy of these inhibitors in a different species and explore the underlying mechanisms through which they may mitigate hepatic steatosis and fibrosis. Our analysis specifically aimed to investigate the effect of selective 11β-HSD1 inhibition on hepatic steatosis and fibrosis, aiming to identify potential underlying pathways.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), M199 medium, RPMI 1640 medium and penicillin-streptomycin solution (PS) 100 × were purchased from Corning (Corning, CA, USA). TB Green Premix Ex *Taq*II was purchased from TAKARA (Shiga, Japan). Key reagents, such as bovine serum albumin (BSA), beta-mercaptoethanol, 4′,6-diamidino-2-phenylindole (DAPI), trisodium citrate, Sirius red, sucrose, streptozotocin, Triton X-100, and ethylene glycolbis(β-aminoethyl ether)-N,N,N′,N'-tetraacetic acid were purchased from Sigma (St. Louis, MO, USA). Eosin, glycogen, hematoxylin, Oil Red O, and heparin were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Test substances

INU-101 [23], ursodeoxycholic acid (UDCA), and OCA were generously provided by Ahngook Pharmaceutical Co., Ltd. (Gyeonggi-do, South Korea). Dosing solutions were prepared by accurately weighing each substance and dissolving it in 0.5 % methylcellulose to ensure uniform dosing.

2.3. Animal experiment design

Male C57BL/6N mice, 8 weeks old and weighing approximately 20–25 g, were obtained from Samtako Bio Korea (Osan, South Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University, Cheongju, Republic of Korea (ethical approval no. CBNUA-1203-18-02). The animals were housed in a pathogen-freewith environmental conditions, including temperature (maintained at 21 ± 2 °C), humidity (kept at 50 % ± 10 %), lighting (controlled on a 12-h artificial light and dark cycle), carefully regulated.

The mice were randomly assigned into different dietary groups: an (FFD) group (40 % calories from fat, 0.2 % cholesterol; RD Western Diet, Open-Source Diets plus fructose 23.1 g/L and glucose 18.9 g/L added to the drinking water) and a normal chow diet

group. Each group consisted of 5–8 mice to ensure high statistical power. The mice had ad libitum access to food and water throughout the 25-week study period. During the last 6 weeks, mice in the FFD group were further subdivided and treated with INU-101 (50 mg/kg), UDCA (185 mg/kg), OCA (30 mg/kg), or vehicle, administered once daily via oral gavage. Dosages were selected based on previously published experimental models to match therapeutic levels observed in clinical settings [23–25].

2.4. Cell lines

Hep3B cells and HepG2 cells were purchased from the American Type Culture Collection (HB-8064 and HB-8065, respectively, ATCC, Manassas, VA, USA). LX-2 cells were obtained from Sigma-Aldrich (SCC064, USA). Hep3B and HepG2 cells were cultured in DMEM supplemented with 10 % FBS and 1 % PS, while LX-2 cells were maintained in DMEM supplemented with 2 % FBS and 1 % PS. All cells were cultured at 37 °C in a humidified atmosphere of 5 % CO2. Moreover, all cell experiments were performed at 70–80 % confluency in 2–5 different wells per day.

2.5. Isolation of primary hepatocytes, hepatic stellate cells (HSCs) and Kupffer cells (KCs)

Primary hepatocytes were isolated by anesthetizing mice with Zoletil (30 mg/kg; Virbac, Carros, France) followed by insertion of a 24G catheter into the inferior vena cava (IVC) for perfusion. The liver was perfused with 30 mL EGTA solution (Sigma-Aldrich) maintained at 37 °C using a Masterflex L/S easy-load II (Cole-Parmer Instrument Co., Vernon Hills, IL, USA). Following this, the liver was perfused with 75 mL enzyme buffer containing collagenase type I (650 μg/mL; Worthington Biochemicals, Lakewood, NJ, USA) and collagenase P (50 μg/mL; Roche, Mannheim, Germany). Subsequently, the liver was dissected, minced, filtered through a 100-μm filter, and washed twice with enzyme buffer. Then, hepatocytes were isolated and cultured in a complete M199 medium (Corning).

To isolate HSCs and KCs from wild type (WT) mice, the digested hepatocyte suspension was centrifuged at 57×*g* for 1 min, and the supernatant was collected and centrifuged at 918×*g* for 10 min to obtain a cell suspension. The cell suspension was gently layered with 70 % Percoll and centrifuged at 2066×*g* for 20 min under an off-brake condition. The isolated HSCs were resuspended in DMEM. Non-parenchymal cells (NPCs) were collected, and KCs were isolated using MojoSort™ Streptavidin Nanobeads (BioLegend, San Diego, CA, USA) [26]. Isolated KCs were resuspended in RPMI 1640 medium (Corning).

2.6. Serum and hepatic biochemistry

Blood samples were collected using a cardiac puncher; serum was separated by centrifugation (4000×*g*, 15 min); and the activity levels of alanine aminotransferase (ALT) and cholesterol were analyzed using Green Cross Lab Cell Corp (Gyeonggi-do, South Korea). Tissue triglyceride levels were measured using a Triglyceride Detection Kit (Cat. No. BM-TGR-100, BioMax, Korea) according to the manufacturer's instructions.

2.7. Histological analysis

Liver tissues were excised from the mice, preserved in formalin, and embedded in paraffin. To assess collagen deposition, liver sections were treated with Sirius red solution (Cat. No. 24901-500, Thermo Fisher Scientific, Waltham, MA, USA). To quantitatively evaluate fibrotic regions, complete liver sections were imaged at a 200 × magnification using DMi8 (Leica, Germany), and the Sirius red-positive areas were quantified using LAS X (Leica, Germany) and ImageJ (NIH, USA) software.

2.8. Quantitative reverse transcription polymerase chain reaction

Cell and liver tissue samples were lysed using RiBoEx (Cat. No. 301-001, GeneAll Biotechnology, Seoul, South Korea) and total RNA was reverse transcribed to complementary DNA using the PrimerScriptTM RT kit (Cat. No. RR037A, TAKARA, Shiga, Japan) with a genomic DNA scavenger (Cat. No. RR037A, TAKARA, Shiga, Japan). Polymerase chain reaction was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Target gene expression was normalized to that of 3-phosphoglycerate dehydrogenase (GAPDH; internal control). The specific primer sequences are listed in Supplementary Table 1.

2.9. Enzyme-linked immunosorbent assays

Hep3B cells and hepatocytes were seeded in 12-well plates at a density of 2 × 10^5 cells/mL in DMEM containing 10 % FBS, followed by a 12-h incubation period. The medium was then replaced with fresh medium (without FBS) supplemented with different concentrations of cortisone and INU-101. The medium was incubated for 24 h at 37 °C and then collected for the required assays. Detection of cortisol was performed using enzyme-linked immunosorbent assay kits (Cat. No. ab108665, Abcam, Cambridge, UK).

2.10. Cytotoxicity

Hep3B cells and extracted primary mouse hepatocytes were inoculated in 12-well (2×10^5 cells/well) plates with or without collagen coating and cultured in M199 medium and DMEM with 10 % FBS and 1 % antibiotic antifungal agent (Gibco, NY, USA). After 12 h, the medium was replaced with fresh supplemental medium without FBS. Subsequently after 24 h of incubation, the cultures were

collected for the cytotoxicity assay (Cat. No. BCT-LDHP500 Biomax, Seoul, South Korea).

2.11. Mitochondrial reactive oxygen (mtROS) and apoptosis assays

Mitochondrial superoxide generation was assessed utilizing MitoSOX Molecular Probes (Cat. No. M36008, Invitrogen, Carlsbad,

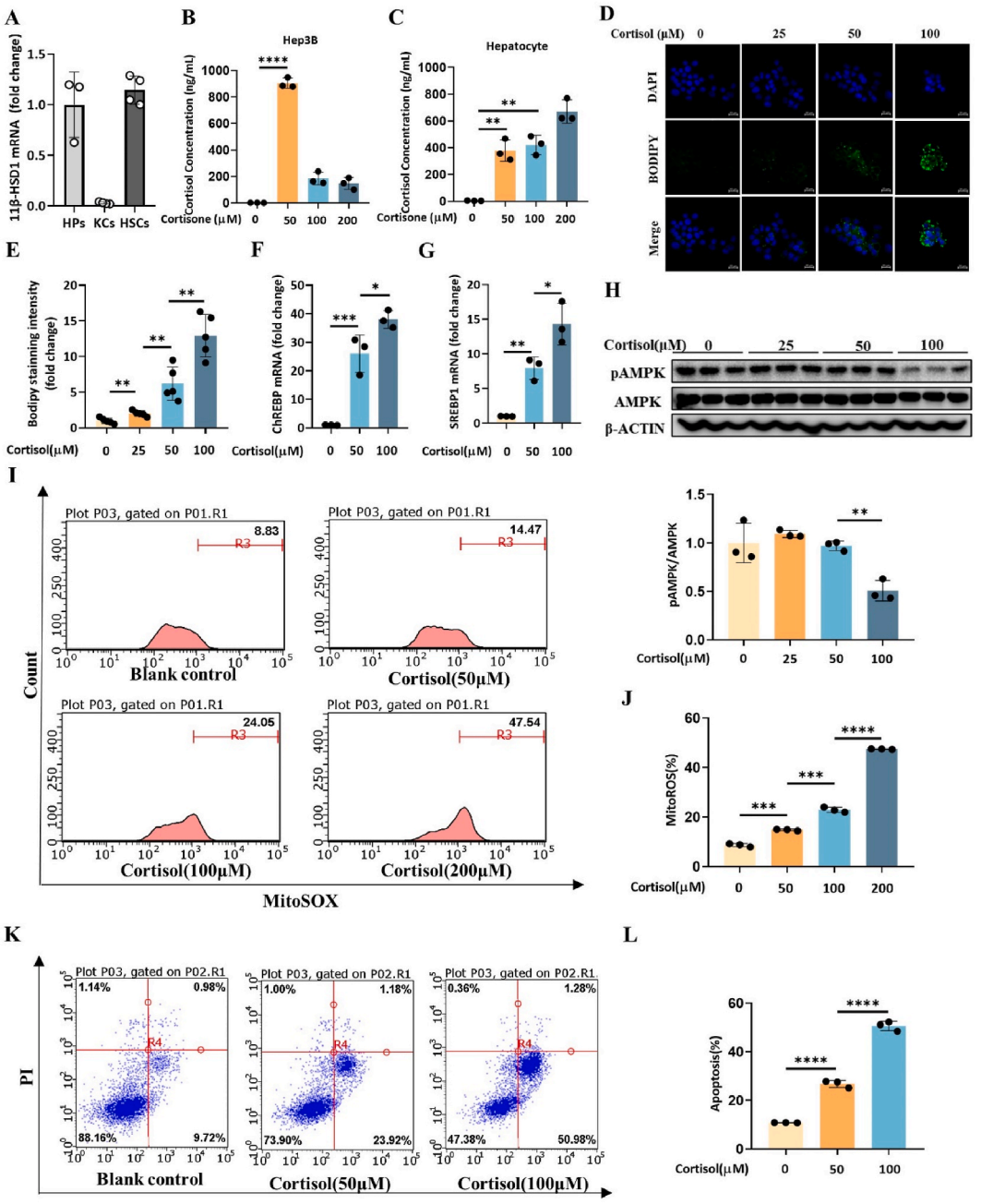

Fig. 1. Cortisol induces hepatic lipid accumulation and subsequent cell death (A) 11βHSD1 mRNA expression in primary hepatocytes, KCs, and HSCs. (B–C) ELISA for detecting conversion of cortisone to cortisol in Hep3B cells and primary hepatocyte. (D–E) BODIPY staining and quantification of cortisol in HepG2 cells. (F–G) mRNA expression of SREBP1 and ChREBP in Hep3B cells treated with cortisol. (H) Quantification of AMPK and p-AMPK protein levels in Hep3B cells following cortisol treatment. The original image was provided in Figure S3 (I-J) Flow cytometry analysis of mitochondrial ROS production in Hep3B cells after cortisol treatment. (K–L) Flow cytometry analysis of apoptosis in Hep3B cells after cortisol treatment. Relative mRNA expression levels were normalized to mouse GAPDH levels. The data are expressed as means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

CA, USA). After treating the cells with cortisol, cortisone, and INU-101, they were rinsed twice with phosphate-buffered saline (PBS) and subsequently exposed to MitoSOX at 37 °C for 30 min. Guava® easyCyte HT Flow Cytometer (Millipore, Darmstadt, Germany) was employed to evaluate the levels of mtROS.

Apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (K29100; Labiskoma, Seoul, Korea). In brief, treated cells (5×10^5) were collected by centrifugation, washed twice with cold PBS, and resuspended in 500 μL of 1 × Annexin V binding buffer containing 1.25 μL of Annexin V-FITC. After incubation for 15 min at 68°F–77 °F, the cells centrifuged for 5 min at room temperature, the supernatant was discarded, and then the cells were washed with 0.5 mL of cold 1 × binding buffer. Next, 10 μL of PI was added to the cell suspension, and apoptosis was analyzed using flow cytometry.

2.12. Western blot analysis

Treated cells were rinsed twice with PBS, harvested, and then subjected to two rounds of ice-cold lysis using radioimmunoprecipitation assay buffer and, a sonicator. Following this, the lysates from both cells and tissues were centrifuged at 28,300×*g* at 4 °C for 15 min. To determine protein concentration in the supernatant, a BCA Protein Assay Kit from Thermo Fisher Scientific was utilized. Subsequently, equivalent quantities of proteins were separated using 10 % or 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5 % skim milk for 1 h at room temperature. Thereafter, they were incubated overnight with primary antibodies (refer to Supplementary Table 2) at 4 °C,

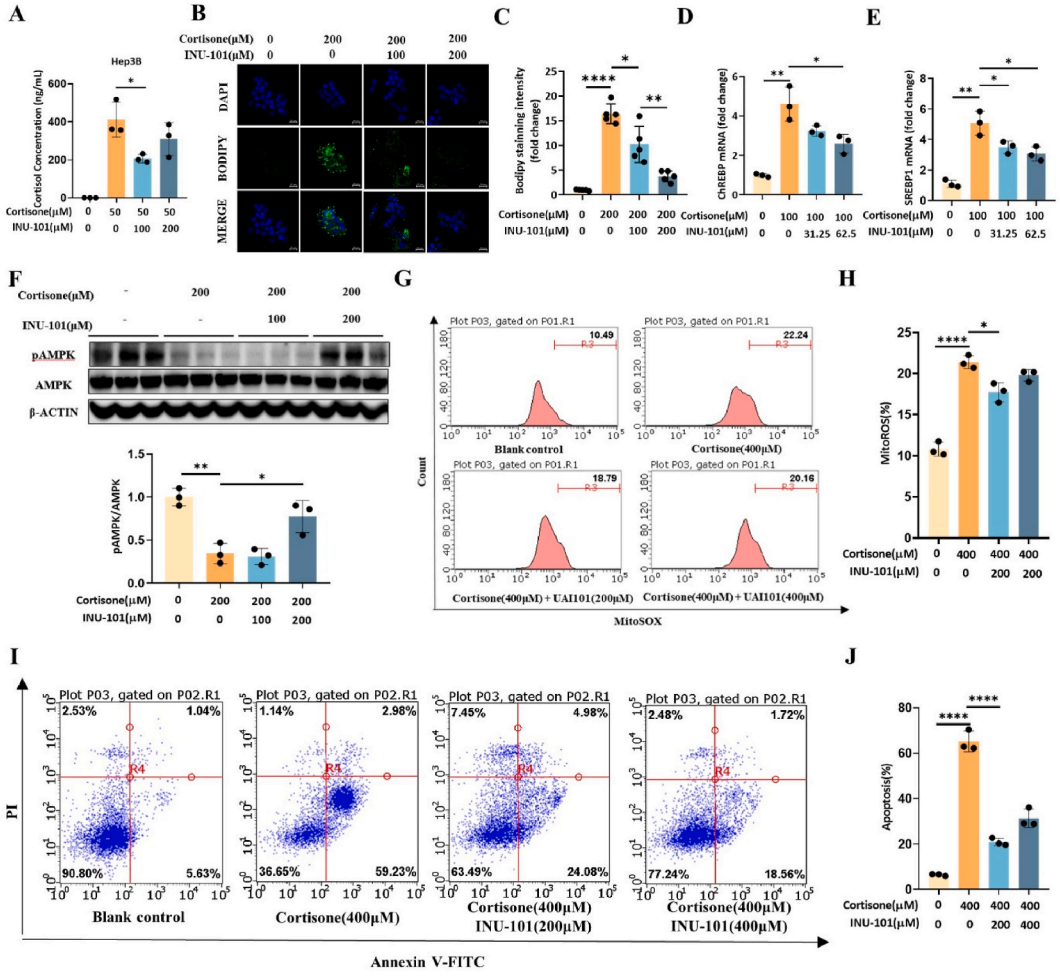

Fig. 2. Blockade of 11β-HSD1 prevents cortisone-mediated lipid accumulation and subsequent cell death (A) ELISA showing cortisone conversion to cortisol following co-treatment with INU-101 in Hep3B cells. (B–C) BODIPY staining and quantification of cortisone following co-treatment with INU-101 in HepG2 cells. (D–E) SREBP1 and ChREBP mRNA expression following co-treatment of cortisone with INU-101 in Hep3B cells. (F) Quantification of AMPK and p-AMPK protein levels in Hep3B cells following cortisone and INU-101 treatment. The original image was provided in Figure S4 (G-H) Flow cytometry analysis of mitochondrial ROS production in Hep3B cells after cortisone and INU-101 treatment. (I–J) Flow cytometry analysis of apoptosis detection in Hep3B cells after cortisone and INU-101 treatment. Relative mRNA expression levels were normalized to mouse GAPDH levels. The data are expressed as means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

and then with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. All antibodies were diluted in Tris-buffered saline/Tween containing 2 % BSA. The signals were visualized using West-Q Pico Dura ECL Solution from GenDEPOT (Baker, TX, USA). The intensity of the bands was quantified using ImageJ software, and the results were normalized to those obtained for β-actin.

2.13. Boron dipyrromethene (BODIPY) staining

To assess fatty acid uptake using BODIPY lipid probes, podocytes were exposed to 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (10 μg/mL, BODIPY 500/510 C1, C12; Invitrogen) and DAPI for 1 h at 37 °C. Next, the cells were washed three times with PBS and promptly imaged using a Super resolution Confocal Laser Scanning Microscope (LSM 980; Carl Zeiss, Germany).

2.14. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). For comparisons among multiple groups, one-way analysis of variance (ANOVA) was used, followed by Tukey's post hoc test to identify significant differences between groups. The threshold for statistical significance was set at $P < 0.05$. The sample size for each experimental group was determined based on power analysis to ensure sufficient sensitivity to detect biologically meaningful differences.

3. Results

3.1. Cortisol induces hepatic lipid accumulation and subsequent cell death

To investigate the effects of cortisol on the liver, we first confirmed the high expression of 11β-HSD1, a gene whose product

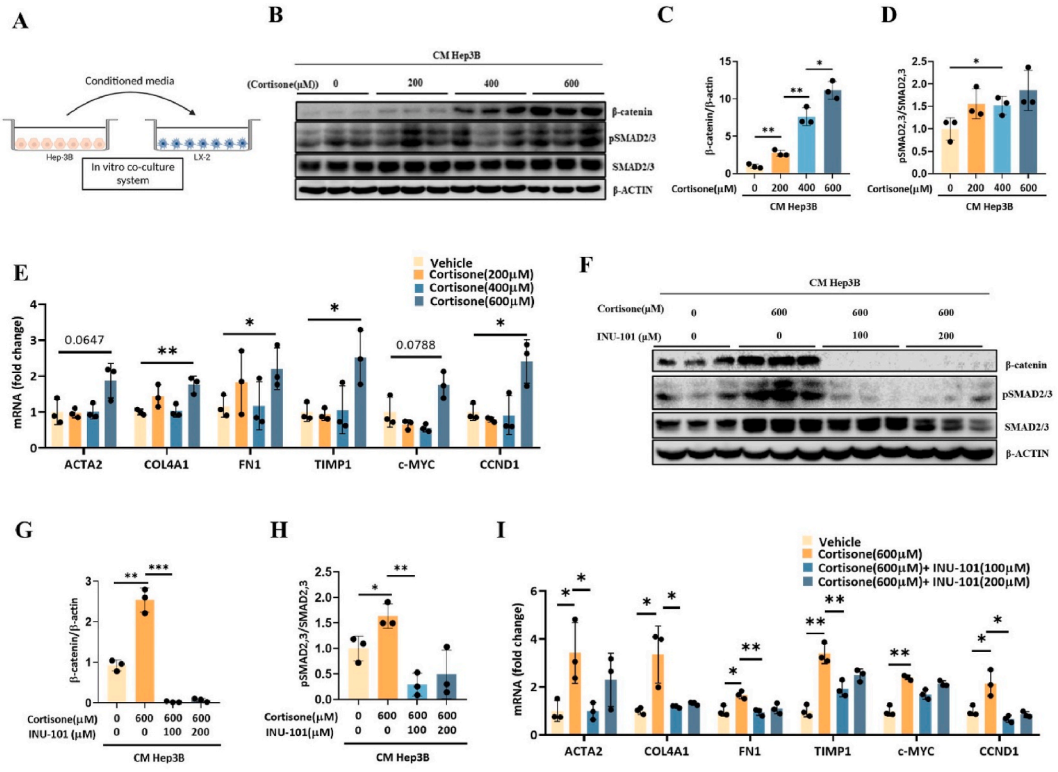

Fig. 3. Cortisol signaling links hepatocyte-derived DAMPs to HSC activation (A) Schematic co-culture system. (B) Protein levels of β-catenin, p-SMAD2/3, and SMAD2/3 in LX2 cells after cell supernatant treatment. The original image was provided in Figure S5 (C-D) TQuantitative analysis of β-catenin/actin and p-SMAD2/3/SMAD2/3. (E) mRNA expression of pro-fibrosis- and proliferation-related genes (including *ACTA2*, *COL4A1*, *FN1*, *TIMP1*, *C-MYC* and *CCND1*) in LX2 cells after cell supernatant treatment. (F) Protein levels of β-catenin, p-SMAD2/3, and SMAD2/3 in LX2 cells after co-treatment with supernatant and INU-101. The original image was provided in Fig. S6. (H) Quantification of β-catenin, p-SMAD2/3, and SMAD2/3. The original image was provided in Fig. S6. (I) mRNA expression of pro-fibrosis- and proliferation-related genes (including *ACTA2*, *COL4A1*, *FN1*, *TIMP1*, *C-MYC* and *CCND1*) in LX2 cells after co-treatment with supernatant and INU-101. Relative mRNA expression levels were normalized to mouse GAPDH levels. The data are expressed as means ± sem. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

responsible for converting cortisone to cortisol, in hepatocytes and HSCs (Fig. 1A). We also demonstrated that cortisone could be efficiently converted to cortisol in primary hepatocytes and Hep3B cells (Fig. 1B and C). BODIPY staining analysis revealed that cortisol induced hepatic lipid accumulation (Fig. 1D and E) and upregulated the mRNA expression of lipogenesis-related genes, such as carbohydrate-responsive element-binding protein (*ChREBP*) and sterol regulatory element-binding protein 1 (*SREBP1*) (Fig. 1F and G). Phosphorylated AMP-activated protein kinase (p-AMPK) plays a central role in the complex process of cellular lipid metabolism [27]. Activation of p-AMPK inhibits lipid overproduction and thus prevents lipid accumulation in hepatocytes. However, cortisol treatment led to a reduction in AMPK phosphorylation in hepatocytes (Fig. 1H), resulting in excessive lipid storage. Excessive lipid accumulation is associated with increased ROS production, eventually leading to hepatocyte injury and death [28]. Our results were consistent with these findings, with cortisol-induced ROS production (Fig. 1I and J) leading to increased hepatocyte apoptosis (Fig. 1K, L, and S1A). These findings align with previous studies indicating that dysregulated hepatic lipids and increased oxidative stress can induce hepatic cell death under metabolic stress conditions [29]. Our results suggest that cortisol treatment decreases AMPK phosphorylation, promotes lipid accumulation, and leads to excessive ROS production, resulting in hepatocyte death.

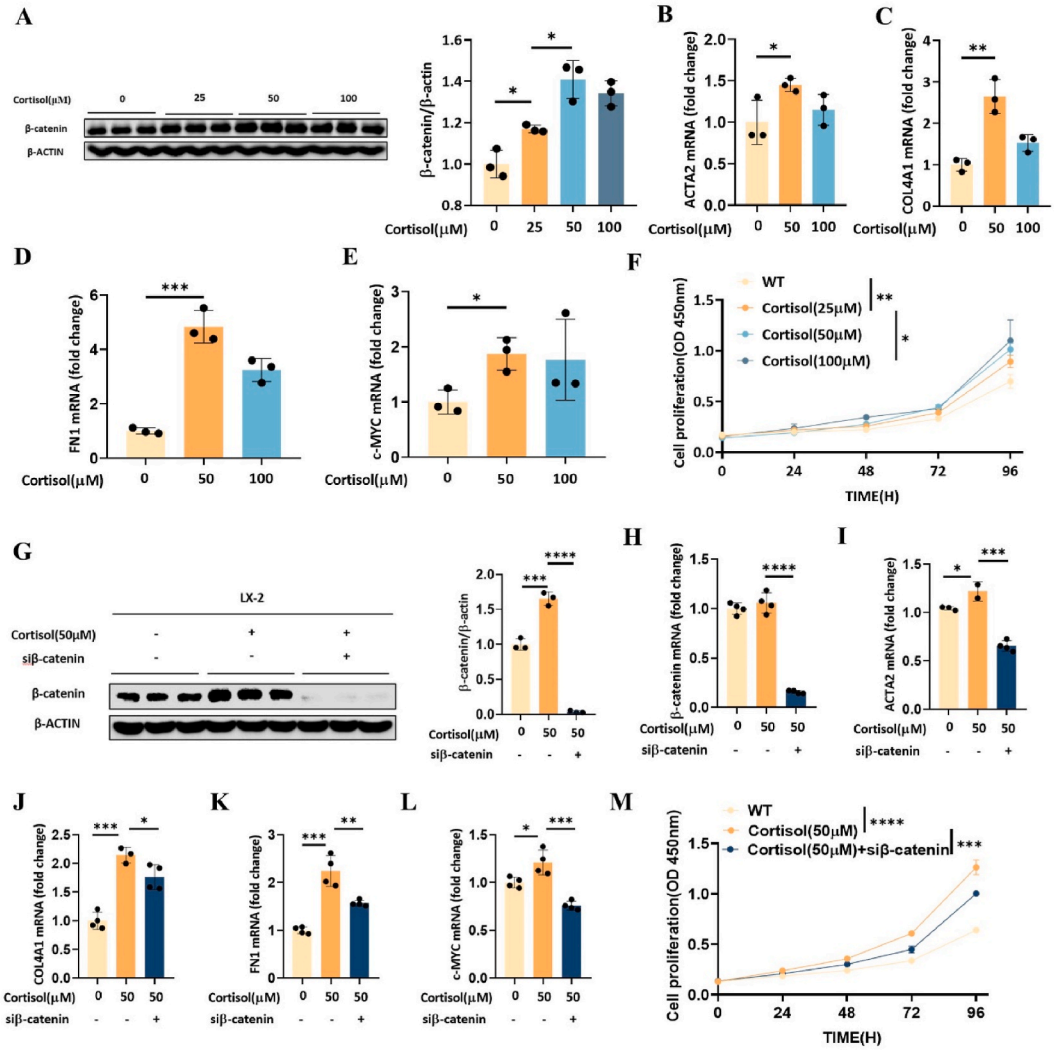

Fig. 4. 11β-HSD1 mediates cortisol-induced HSC activation by regulating β-catenin signaling (A) Protein level of β-catenin in cortisol-treated LX2 cells. The original image was provided in Figure S7 (B-E) mRNA expression of pro-fibrosis- and proliferation-related genes (including *ACTA2*, *COL4A1*, *FN1*, *C-MYC* and *CCND1*) in LX2 cells. (F) Cell proliferation assay of LX2 after treatment with different concentrations of cortisol. (G) Protein levels of β-catenin. The original image was provided in Figure S8 (H-L) mRNA expression of pro-fibrosis- and proliferation-related genes in LX2 cells after treatment with cortisol or cortisol combined with β-catenin knockdown. (M) Cell proliferation assay of LX2 cells after treatment with cortisol or cortisol combined with β-catenin knockdown. Relative mRNA expression levels were normalized to mouse GAPDH levels. The data are expressed as means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.2. Blockade of 11β-HSD1 prevents cortisone-mediated lipid accumulation and subsequent cell death

11β-HSD1 inhibitor INU-101 effectively reduced the conversion of cortisone to cortisol in hepatocytes without exhibiting drug toxicity (Fig. 2A–S1B, and S1C). Furthermore, BODIPY staining revealed that INU-101 efficiently alleviated lipid accumulation (Fig. 2B and C) and downregulated the expression of cortisone-induced lipogenic genes, including ChREBP and SREBP1 (Fig. 2D and E). INU-101 treatment significantly reduced cortisone-induced mtROS production and cell death by increasing AMPK phosphorylation (Fig. 2F–J, S1C, and S2A-C). These findings are similar to those of previous studies showing that inhibiting 11β-HSD1 improves lipid metabolism and reduces cellular stress markers in hepatic cells, highlighting the therapeutic potential of targeting this enzyme in liver diseases [18]. Our findings indicate that INU-101 inhibits the conversion of cortisone to cortisol in hepatocytes by targeting 11β-HSD1, thereby significantly suppressing lipogenesis and hepatocyte apoptosis.

3.3. Cortisol signaling links hepatocyte-derived DAMPs to HSC activation

During lipid overload, hepatocytes may release damage-associated molecular patterns (DAMPs) as part of the cellular interactions in liver diseases [30]. Cortisone promotes hepatocyte death and lipid accumulation through its conversion to cortisol (Fig. 2). To investigate cortisone-mediated interactions between hepatocytes and HSCs, we used a coculture system (Fig. 3A). LX2 cells were treated with conditioned medium (CM) obtained from cortisone-treated hepatocytes with or without 11β-HSD1 inhibition. Our analyses revealed that cell cultures without 11β-HSD1 inhibition exhibited elevated levels of β-catenin protein and phosphorylation of

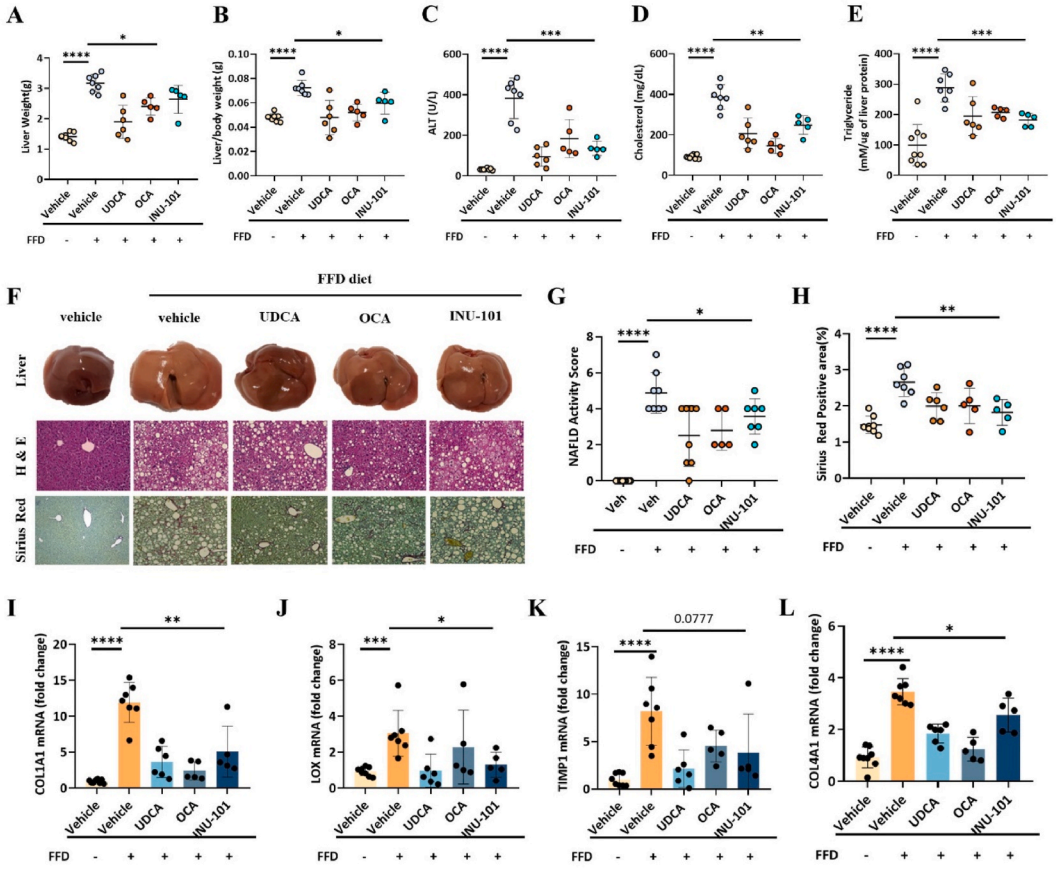

Fig. 5. Pharmacological inhibition of 11β-HSD1 ameliorates FFD-induced MASLD and fibrosis (A) Liver weight of mice fed for 24 -weeks with normal chow diet (NCD), FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (B) Liver-to-body weight ratio of 2mice fed for 4-weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (C) Serum ALT levels of 24 -weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (D) Serum cholesterol levels of 24 -weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (E) Serum triglyceride levels of mice fed for 24-weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (F) Macroscopic observation, H&E staining and Sirius red staining of liver tissues from mice fed for 24-weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (G) NAS scoring of liver tissues from mice fed for 24 -weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101. (H) Quantification of Sirius red staining of liver tissue from mice fed for 24 -weeks with NCD, FFD, FFD + UDCA, FFD + OCA, and FFD + INU-101 liver tissue. (I–L) mRNA expression of pro-fibrosis-related genes, such as *COL1A1*, *LOX*, *TIMP1* and *COL4A1*, in liver tissue. Relative mRNA expression levels were normalized to mouse GAPDH levels. The data are expressed as means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

SMAD2/3 after exposure to CM (Fig. 3B–D). Additionally, we observed increased mRNA expression of fibrosis-related genes and cell proliferation markers (Fig. 3E), including actin alpha 2 (*ACTA2*), collagen type IV alpha 1 chain (*COL4A1*), fibronectin 1 (*FN1*), tissue inhibitor of metalloproteinase 1 (*TIMP1*), cellular myelocytomatosis oncogene (*c-MYC*), and cyclin D1 (*CCND1*). CM with 11β-HSD1 inhibition caused reduced phosphorylation of β-catenin and SMAD2/3 as well as decreased expression of fibrosis and proliferation-related genes (Fig. 3F–I). These results align with the role of DAMPs in mediating HSC activation in injured hepatocytes [31], suggesting that targeting 11β-HSD1 may be offer a therapeutic approach to mitigate liver fibrosis by modulating DAMP-mediated HSC activation.

3.4. 11β-HSD1 mediates cortisol-induced HSC activation by regulating β-catenin signaling

11β-HSD1 is not only highly expressed in hepatocytes but is also abundantly expressed in HSCs (Fig. 1A). We explored the role of cortisol in HSCs and its underlying mechanism. Aberrant activation of β-catenin induces cell proliferation and promotes tissue fibrosis [32]. Notably, cortisol treatment significantly induced the upregulation of β-catenin protein levels (Fig. 4A). Consistent with the induction of *β-catenin*, cortisol treatment also led to an increase in the mRNA expression of fibrosis markers, such as *ACTA2*, *COL4A1*, and *FN1* (Fig. 4B–D). Additionally, *c-MYC* mRNA expression significantly increased after cortisol treatment (Fig. 4E). Furthermore, our findings demonstrated that cortisol substantially increased HSC proliferation (Fig. 4F). To investigate whether cortisol-induced HSC activation is mediated by β-catenin, we conducted knockdown experiments targeting β-catenin and co-treated cells with cortisol. The results revealed that β-catenin knockdown mitigated cortisol-induced HSC activation by reducing the mRNA expression of fibrosis and proliferation-related genes (Fig. 4G–M). These findings are consistent with previous research on the role of β-catenin in fibrosis [33]. Our study indicated that cortisol activates HSC activation via β-catenin.

3.5. Pharmacological inhibition of 11β-HSD1 ameliorates FFD-induced MASLD and fibrosis

We also evaluated the impact of 11β-HSD1 inhibition on the intricate pathogenesis of FFD-induced MASLD and fibrosis. To induce MASLD and fibrosis, we administered the 11β-HSD1 inhibitor INU-101 to mice subjected to a 25-week FFD regimen. Simultaneously, we sought to determine the therapeutic efficacy of INU-101 in comparison to the established therapeutic agents UDCA and OCA, both of which have exhibited clinical effectiveness for MASLD treatment [34]. The administration of the 11βHSD1 inhibitor led to a significant reduction in the liver weight and liver-to-body weight ratio (Fig. 5A and B). In addition, the INU-101 treatment group showed improvements in ALT, total cholesterol, and triglyceride levels (Fig. 5C–E). Collectively, these results indicated a marked attenuation of FFD-induced hepatic injury and inflammation.

Inhibition of 11β-HSD1 also had a broader positive impact, including mitigation of hepatic steatosis, inflammation, and fibrosis induced by FFD. This pivotal observation was confirmed through comprehensive assessments, including HE staining, Sirius red staining, and quantitative Sirius staining analysis (Fig. 5F and G). The histological evaluation revealed strong evidence of a substantial decrease in the metabolic dysfunction-associated fatty liver activity score in the INU-101 treatment group, indicating amelioration of hepatocyte ballooning (Fig. 5H). The therapeutic effects of INU-101 on MASLD and liver fibrosis were comparable to those of UDCA and OCA (Fig. 5I–L), aligning with findings from studies exploring alternative therapeutic pathways for MASLD. These results underscore 11β-HSD1 inhibition as a promising approach for treating MASLD and fibrosis, providing a foundation for its further clinical development [35].

4. Discussion

11β-HSD1 catalyzes the conversion of inactive cortisone to its active form, cortisol. Excessive cortisol levels, which are observed under conditions of stress or inflammation, can potentially contribute to liver fibrosis by promoting oxidative stress, inflammation, and hepatocyte damage [36]. Consequently, inhibiting 11β-HSD1 could balance cortisol level and potentially counteract these detrimental effects [37]. However, the existing literature also suggests that 11β-HSD1 inhibition can exacerbate fibrosis, underscoring the complexity of the molecular interactions and pathways involved [38]. These discrepancies can be attributed to differences in factors, such as experimental models, tissue contexts, and methodologies, highlighting the need for further research, a robust experimental design, and a comprehensive analysis of the underlying mechanisms. In the current study, the importance of 11β-HSD1 inhibition was reflected by its potential to serve as a novel approach for treating liver fibrosis by modulating GC metabolism, affecting HSC activation, and potentially disrupting fibrosis-promoting pathways [39]. Acknowledging and addressing discrepancies in the literature are essential steps toward advancing our understanding and harnessing the therapeutic potential of 11β-HSD1 inhibition in the context of liver disease.

GC activity, which is potentially triggered by factors, such as obesity or stress, can lead to lipid accumulation in various tissues, including the liver [40]. This accumulation is linked with the progression of conditions such as MASLD and its more severe form, MASH. AMPK is a pivotal regulator of cellular energy balance and metabolism [41], and its activation plays a crucial role in mitigating the detrimental effects of excess lipids by facilitating counteractive processes such as enhanced fatty acid oxidation and improved glucose metabolism [42]. In the present study, cortisolimpeded the phosphorylation of AMPK, which in turn promoted the expression of genes involved in lipid synthesis in hepatocytes. This sequence of events occurs through a signaling pathway mediated by AMPK, culminating in lipid accumulation. In addition, the inhibition of 11β-HSD1 successfully reversed the cortisol-induced lipid accumulation, implying that targeting 11β-HSD1 could potentially mitigate the effects of cortisol-induced lipid overaccumulation and its associated complications.

Excessive lipid buildup within liver cells induces lipotoxicity, which triggers oxidative stress and disrupts the equilibrium between ROS production and the body's antioxidant defense mechanisms [43]. The process of lipid peroxidation induced by these ROS plays a pivotal role [44]. The resulting oxidative stress serves as a catalyst for hepatocyte impairment and subsequently leads to the release of DAMPs [45], which in turn activate HSCs and are pivotal drivers of liver fibrosis progression. Our findings offer insights into this multifaceted interplay. We demonstrated that the enzymatic conversion of cortisone to cortisol within hepatocytes triggered heightened lipid accumulation, leading to excessive production of ROS, and culminating in hepatocyte death and the consequent release of DAMPs. This cascade of events was investigated using a co-culture system that allowed, hepatocytes and HSCs interact. DAMPs from hepatocytes induced the activation of β-catenin and the upregulation of P-SMAD2/3 protein expression, which together triggered HSC activation to exacerbate fibrosis. The inhibition of 11β-HSD1 yielded remarkable outcomes: in addition to curtailing the release of DAMPs from hepatocytes, it also resulted in a substantial reduction in HSC activation. Thus, targeting this intricate mechanism may be a prospective avenue for mitigating hepatocyte-derived DAMP-mediated HSC activation and reducing the progression of fibrosis. Our study provides a comprehensive perspective on the intricate relationships of lipid accumulation, oxidative stress, DAMP release, and HSC activation in the context of liver fibrosis, and our findings highlighting the effects of 11β-HSD1 inhibition in relation to cortisone-to-cortisol conversion suggest a potential therapeutic strategy. By inhibiting hepatocyte DAMP release, we effectively disrupted the vicious cycle that fuels HSC activation and fibrosis progression.

Liver fibrosis is a pathological condition characterized by the accumulation of excessive connective tissue within the liver, which is a consequence of persistent injury or inflammation [46]. β-catenin is a key protein intricately involved in various process, such as cell adhesion, signaling, and gene expression [47]. Emerging research has revealed that this multifunctional protein, particularly in its β-linker form, contributes significantly to the intricate web of mechanisms driving liver fibrosis [48]. Central to the fibrotic process are HSCs, whose activation is precipitated by liver injury. Their role in producing excessive extracellular matrix constitutes the foundation for the development of fibrosis [49]. β-catenin signaling appears to orchestrate this activation by inducing the transformation of HSCs to fibrotic myofibroblasts, which drives the progression of fibrosis [50]. Our findings indicated that HSCs contain high levels of 11β-HSD1, and that cortisol, a hormone implicated in stress response, had a significant influence. Cortisol triggers the activation of HSCs by intricately regulating the β-catenin pathway [43]. Our experiments revealed that 11β-HSD1 inhibition can counteract cortisol-induced HSC activation through fine-tuned regulation of β-catenin signaling, highlighting a potential avenue for dampening the impact of HSC-mediated fibrosis.

In conclusion, our findings demonstrate that inhibiting 11β-HSD1 can effectively reduce hepatic steatosis, inflammation, and fibrosis associated with FFD-induced conditions, highlighting its potential as a therapeutic target for MASLD and related liver disorders. By regulating GC metabolism, 11β-HSD1 inhibitors may also benefit other metabolic conditions, such as obesity and diabetes. We recommend advancing these inhibitors into clinical trials for MASLD, exploring combination therapies, and thoroughly evaluating their safety and efficacy to improve the management of liver fibrosis and metabolic syndrome-related liver diseases.

CRediT authorship contribution statement

Hwan Ma: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Guo-Yan Sui:** Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Jeong-Su Park:** Resources, Investigation. **Feng Wang:** Resources, Investigation. **Yuanqiang Ma:** Resources, Investigation. **Dong-Su Shin:** Resources, Investigation. **Nodir Rustamov:** Resources, Investigation. **Jun Sung Jang:** Resources. **Soo Im Chang:** Resources. **Jin Lee:** Writing – review & editing, Investigation. **Yoon Seok Roh:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Ethical statement

All animal experiments and protocols were performed in accordance with the guidelines of the Chungbuk National University, Cheongju, Republic of Korea (ethical approval no. CBNUA-1203-18-02).

Data and code availability statement

Data included in article/supplementary material is referenced in the article.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jun Sung Jang reports equipment, drugs, or supplies was provided by Ahn-Gook Phamaceutical Co., Ltd. Soo Im Chang reports

equipment, drugs, or supplies was provided by Ahn-Gook Phamaceutical Co., Ltd. Jun Sung Jang reports a relationship with Ahn-Gook Phamaceutical Co., Ltd. that includes: employment. Soo Im Chang reports a relationship with Ahn-Gook Phamaceutical Co., Ltd. that includes: employment. INU-101 is under intellectual property rights owned by Ahn-Kook Pharmaceutical Co., Ltd., Korea, and we conducted experiments with the provision of INU-101, UDCA, and OCA. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39534.

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