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Sodium acetate attenuates hepatic fibrosis by modulating EPHB2-mediated signaling in hepatic stellate cells

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ABSTRACT: Hepatic fibrosis (HF), characterized by excessive extracellular matrix (ECM) deposition, is an essential pathological process in chronic liver diseases, with limited therapeutic options. Short-chain fatty acids (SCFAs), produced by the gut microbiota, have demonstrated the ability to regulate inflammation and fibrotic processes. This study assessed the protective roles of SCFAs, including sodium acetate (NaA), sodium propionate (NaP), and sodium butyrate (NaB), against CCl₄-induced hepatic fibrosis in mice. Administration of SCFAs, particularly NaA and NaB, significantly ameliorated CCl₄-induced liver dysfunction by reducing serum ALT and AST levels, improving histopathological architecture, and attenuating lipid deposition and inflammation through inhibition of NF-κB signaling. NaA led to a substantial reduction in collagen deposition, accompanied by downregulation of *Colla1*, *Acta2*, and *Tgf-β1* gene expression. In vitro, NaA also inhibited activation and proliferation of TGF-β1-activated LX2 hepatic stellate cells (HSCs), by downregulating ECM markers (*FNI*, *COL1A1*) and modulating the mRNA levels of *MMP-9* and *TIMP-1*. Data-independent acquisition (DIA) -based proteomic analysis identified 25 differentially expressed proteins (DEPs) reversed by NaA treatment, with ephrin receptor B2 (EPHB2) identified as a key target. Combined treatment with NaA and the EPHB2 inhibitor AWL-II-49-7 did not further inhibit LX2 cells activation, but resulted in a greater suppression of EPHB2 protein expression, supporting EPHB2 as a key mediator of the antifibrotic effects of NaA. These findings highlight the anti-fibrotic potential of SCFAs, particularly NaA, through EPHB2-mediated pathways, providing a novel potential therapeutic strategy for HF.

Keywords: Sodium acetate, Short chain fatty acid, Fibrosis, Hepatic stellate cells, EPHB2

1. Introduction

Hepatic fibrosis (HF) affects over 800 million individuals globally and represents a major public health concern [1,2], with its prevalence steadily increasing alongside rising rates of metabolic and chronic liver diseases. Each year, more than two million people die as a result of HF or its complications. Without timely intervention, approximately 75%–80% of patients may progress to cirrhosis [3], liver failure, or hepatocellular carcinoma. The high incidence of HF is primarily driven by a range of chronic liver injuries, including metabolic dysfunction-associated steatotic liver

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disease (MASLD), persistent viral hepatitis (especially Hepatitis B Virus and Hepatitis C Virus), toxic liver damage from alcohol or drugs, and autoimmune liver diseases [2]. At the molecular level, HF is characterized as a persistent wound-healing response triggered by sustained liver injury, characterized by the activation of hepatic stellate cells (HSCs) and excessive accumulation of extracellular matrix (ECM), particularly type I collagen (COL1A1), leading to fibrotic scarring [4]. This process is orchestrated by multiple interacting factors, including chronic hepatocyte damage, epithelial/endothelial barrier disruption, infiltration of bone marrow-derived inflammatory cells, release of cytokines like transforming growth factor- β 1 (TGF- β 1), and activation of collagen-producing myofibroblasts [5]. Despite growing insights into these mechanisms, there is currently no approved antifibrotic therapy, and reversal of fibrosis remains a slow and difficult process [6]. For advanced stages, including cirrhosis, liver transplantation remains the only effective option, underscoring the urgent need for treatments capable targeting fibrosis regression.

The gut and its microbiota form a bidirectional regulatory axis with the liver via the portal vein, forming the gut–liver axis that plays a crucial role in maintaining hepatic homeostasis. The liver is constantly exposed to a range of gut-derived microbial metabolites, including short-chain fatty acids (SCFAs), ethanol, bile acids, and pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), flagellin, and peptidoglycan, flagellin [7]. Under normal conditions, these signals support metabolic and immune balance. However, dysbiosis and enhanced intestinal permeability permit the entry of harmful microbial products into the portal bloodstream, triggering hepatic immune responses (e.g., TLR4 signaling) and contributing to steatosis, inflammation, and fibrosis [8]. These interactions underscore the pivotal role of the gut microbiota and its metabolites in the development of MASLD and HF. Colonic microbiota ferment indigestible dietary fibers to produce short-chain fatty acids (SCFAs), which serve as important microbial metabolites. In the human intestine, acetate, propionate, and butyrate represent the dominant SCFAs, together making up about 95% of total SCFAs [9]. Beyond supplying up to 70 % of colonocyte energy needs [10], SCFAs function as signaling molecules that regulate inflammation and cellular homeostasis via both receptor-dependent G protein-coupled receptor 41/43 (GPR41/43) and receptor-independent mechanisms [11]. In the liver, they are pivotal modulators of metabolism: improving lipid handling, enhancing insulin sensitivity, and dampening inflammatory pathways [12]. In metabolic liver disease models MASLD [13,14], SCFAs ingestion can inhibit liver steatosis by activating AMP-activated protein kinase (AMPK) signalling and upregulating fatty acid oxidation genes [15–17] and downregulating fatty acid synthesis enzymes to correct lipid metabolism in hepatocytes [16]. Additionally, SCFAs can suppress key pro-inflammatory cytokines and chemokines, such as interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α) and C-X-C motif chemokine ligand 2 (CXCL2) to exert an anti-inflammatory effect [18,19]. Importantly, individual SCFAs exhibit different mechanisms of action and anti-inflammatory properties, which may be associated with dose and dependent on SCFA type. For instance, sodium acetate (NaA) can dose-dependently regulate the inflammatory response of macrophages [15], while sodium propionate (NaP) primarily alleviates hepatic steatosis and improves glucose homeostasis [19], while sodium butyrate (NaB) promotes β -oxidation and inhibits liver inflammation [18]. Therefore, the potential effect and effective dosage of different short chain fatty acids on HF, especially drug-induced HF still need to be studied.

HSCs, the primary precursors of hepatic myofibroblasts, serve as the main effector cells in hepatic fibrosis. Their activation and subsequent ECM production are regulated by multiple pro-fibrotic signaling pathways, including the TGF- β /Smad cascade^[20], Platelet-Derived Growth Factor (PDGF)-induced PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways^[21], Hippo/YAP signaling^[22], and reactive oxygen species-mediated stress, as well as interactions with immune cells (Kupffer cells, B cells, monocyte-derived macrophages, and T cells), liver sinusoidal endothelial cells, cholangiocytes, which release the inflammatory and fibrotic factors TGF- β 1, PDGF, TNF- α , CXCL-2, and IL-1 β to trigger the transdifferentiation of HSCs into collagen-secreting myofibroblasts. Additionally, in diverse models of hepatic steatosis, SCFAs, especially NaB, potently inhibit HSCs activation and suppress fibrosis markers. In rat models of metabolic dysfunction-associated steatohepatitis, NaB reduces fibrosis by blocking HSC activation through the miR-155-5p/SOCS1/PDGF signaling axis^[23]. As a histone deacetylase (HDAC) inhibitor, NaB also suppresses LX2 cells (Human Hepatic Stellate Cell Line) proliferation and downregulates fibrogenic genes, including *ACTA2* (α -smooth muscle actin, α -SMA) and *COL1A1*^[24]. Most studies focus on fatty liver-related or secondary fibrosis, with few investigating primary or toxin-induced (chemically induced) HF. This highlights the need for research into SCFAs in chemically induced HF. Furthermore, the specific target cells of SCFAs in the inhibition of fibrosis remain unclear and require further research.

In this study, we present the first study comparing three different doses of SCFAs in a carbon tetrachloride (CCl₄)-induced HF model to explore their therapeutic potential beyond metabolic disorders. We used the LX2 human hepatic stellate cell model combined with data-independent acquisition (DIA) proteomics to analyze the mechanism of action and targets of SCFAs to inhibit TGF- β 1-activated HSCs, and validated the targets of action using inhibitors. This may support the development of microbiota-derived metabolites as novel antifibrotic agents and promote the advancement of targeted therapies for hepatic fibrosis.

2. Materials and methods

2.1 Animals, experiment design and ethics

Male C57BL/6 J mice (6–8 weeks, 18–22 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were kept in a specific pathogen-free (SPF) facility under controlled temperature and humidity (40–60%), maintained on a 12-hour light/dark cycle, with unrestricted access to standard chow and water. All animal experiments were performed in compliance with institutional health guidelines and approved by Animal Research Ethics Committee of China Agricultural University (Approval number: Aw90801202-4-1). After a 7-day acclimation period, mice were randomly assigned to eight groups (n=8 per group): (1) Control group (CON) receiving intraperitoneal (i.p.) injections of corn oil vehicle (3 times/week) with concurrent oral saline administration; (2) Model group (MOD) administered CCl₄ (0.5 mL/kg of a 10% (v/v) CCl₄ solution in olive oil, i.p., 3 times/week) plus oral saline; (3) Six treatment groups treated with equivalent CCl₄ regimen plus daily oral administration of NaA, NaP, or NaB at low- (250 mg/kg/day) or high-dose (1000 mg/kg/day) levels. All SCFA solutions were dissolved in sterile

0.9% saline, sterilized using 0.22 μm filters, and delivered to mice by oral gavage. After 6 weeks of intervention, animals underwent a 12-hour fast before euthanasia. Blood was collected at sacrifice, allowed to clot, and then centrifuged at 1,500 g for 10 minutes at 4 °C to isolate serum. The livers were dissected, weighed, and tissue specimens were collected for additional analyses.

2.2 Cell Culture and Treatment

The LX2 cell (immortalized human hepatic stellate cell line) was obtained from the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented 10% (v/v) fetal bovine serum (FBS; Gibco), along with 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin (Beyotime, Beijing, China). Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 .

For analysis of SCFA-mediated modulation of TGF- β 1-induced fibrogenesis, LX2 cells ($1 \times 10^6/\text{well}$) were cultured in six-well plates (Corning) with 2 mL DMEM. When cultures reached 70–80% confluence, cells were incubated in serum-free DMEM for 12 hours. The control group received fresh DMEM, while treatment groups were exposed to DMEM supplemented with 5 ng/mL TGF- β 1 (Sino Biological, Beijing, China) and NaA (0.1 or 1 mM) for 24 h. Selection of NaA concentrations was guided by established toxicological findings in fibrogenesis models using LX2 cells.

To investigate the regulatory impact of NaA on Ephrin type-B receptor 2 (EPHB2) expression in LX2 cells, six experimental groups were designed: (1) control, (2) model (stimulated with TGF- β 1), (3) NaA treatment (0.1 mM), (4) EPHB2 inhibitor alone (0.5 μM AWL-II-49-7), (5) AWL-II-49-7 combined with TGF- β 1, and (6) AWL-II-49-7 combined with NaA. All groups, except the control and inhibitor-alone groups, were treated with TGF- β 1 (5 ng/mL) to induce fibrotic activation. For treatments involving the inhibitor, following serum starvation, cells were pretreated with AWL-II-49-7 (0.5 μM) for 1 hour, and subsequently incubated with TGF- β 1 (5 ng/mL) and/or NaA (0.1 mM) for 24 hours. Following treatment, cells were collected for subsequent Western blotting and qPCR analysis of fibrosis-associated genes.

2.3 Cell viability

Cell viability was assessed using the Cell Counting Kit-8 assay (CCK8, Beyotime, Shanghai, China) assay. LX2 cells were plated at a density of 5×10^4 cells per well in flat-bottomed 96-well plates (Corning). For NaA cytotoxicity testing, cells were treated with various concentrations of sodium acetate (0.1, 1, 2, or 5 mM) for 24 hours. To evaluate the effect of the EPHB2 inhibitor AWL-II-49-7, cells were exposed to increasing concentrations (0.01, 0.1, 0.5, 1, or 2 μM) for 24 hours. The optimal dose of AWL-II-49-7 was then combined with a gradient of NaA (0.01, 0.1, 0.5, or 1 mM) to determine the most suitable concentration for combination treatment. After each treatment, CCK8 reagent was added and cells were incubated for 2 hours, followed by absorbance measurement at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). The cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{450} (\text{Treated}) - \text{OD}_{450} (\text{Blank})}{\text{OD}_{450} (\text{Control}) - \text{OD}_{450} (\text{Blank})} \times 100\%$$

2.4 Western blot

Western blot analysis was performed on both frozen liver tissue and cultured cell samples. Primary antibodies used for membrane incubation included α -SMA, β -actin, Nuclear Factor Kappa B Subunit 1 (p65), Phosphorylated p65 (P-p65, CST, Danvers, MA, USA), and EPHB2 (ABclonal Technology, WuHan, China). After incubation with goat anti-rabbit HRP-conjugated secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China, 1:5,000 dilution) at room temperature for 1 hour, protein bands were visualized using the Luminata Forte Enhanced Chemiluminescence Kit (Millipore), imaged with an Amersham Imager 600 (Amersham Biosciences, Sweden), and quantified using Image Lab software.

2.5 Biochemical assays

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured as markers of liver function with a Mindray BS-350E automated biochemical analyzer (Mindray, Shenzhen, China) following the manufacturer's protocols. All biochemical parameters were expressed as U/L.

2.6 Histological analysis

Liver tissues were excised, with a portion fixed in 4% paraformaldehyde and paraffin-embedded for histological analysis, including H&E, Masson's trichrome, Sirius Red, and immunohistochemical (IHC) staining; another portion was snap-frozen in OCT, snap-frozen, and sectioned at 8 μ m for Oil Red O staining. Quantification of stained areas was performed with Image-Pro Plus software (Media Cybernetics). For IHC analysis, liver sections were first incubated at 4°C overnight with anti-F4/80 (Abcam, USA), then with secondary antibody at 37°C for 1 hour. Diaminobenzidine substrate with hydrogen peroxide was employed for chromogenic detection. Random microscopic fields were examined and quantified under an optical microscope.

2.7 DirectDIA Proteomic Analysis

Sample preparation

Proteins from LX2 cells (n=3) were extracted and quantified using the BCA assay, reduced with dithiothreitol (95 °C, 5 min), alkylated with 50 mM iodoacetamide at room temperature in the dark for 30 minutes, and digested with trypsin (37 °C, overnight). Dried peptides were reconstituted in 0.1% formic acid, spiked with the iRT standard, and analyzed by LC–MS/MS.

nanoLC-MS/MS analysis

For each sample, approximately 500 ng of total peptides were resolved by a nano - Ultra Performance Liquid Chromatography system (Vanquish neo) and then coupled to a mass spectrometer (Astral) with a nano - electrospray ion source for data acquisition. Chromatographic separation was performed on a reversed - phase column EASY - Spray™ HPLC (150 μ m $\times$ 15 cm, Thermo Scientific, USA). The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in 80% acetonitrile (B). Prior to analysis, the column was equilibrated with 96% phase A, and samples were automatically injected. Subsequently, the samples were separated by a gradient elution with a duration of 6.9 min.

DIA was carried out in profile and positive mode with an Orbitrap analyzer. MS1 spectra were acquired at a resolution of 240,000 over an m/z range of 380–980. For MS2, the m/z range was set to 150–2000 with a 2 m/z isolation window. Higher-energy collisional dissociation (HCD) was applied using a normalized collision energy (NCE) setting of 25%.

DIA-NN database search

DIA-NN software (1.8.1) was utilized to process the raw MS files. MS spectra lists were searched against the reviewed UniProt Homo sapiens (Swiss-Prot, taxon ID: 9606, release 2024_05, downloaded May 2024) protein database, Carbamidomethyl [C] of cysteine was set as a fixed modification, while oxidation of methionine and N-terminal acetylation were specified as variable modifications. Trypsin was specified as the protease, allowing up to two missed cleavages. Both PSM and peptide identifications were filtered at a 1% false discovery rate. Peptide identification allowed precursor and fragment mass deviations of up to 20 ppm. All other parameters were kept at their default settings.

Data Analysis and Bioinformatics Analysis

Raw DIA proteomics data were processed as follows. Proteins identified by at least one unique peptide were retained for further analysis. Missing values were imputed using the half-minimum method, as appropriate for DIA data. After preprocessing, the quantified protein intensity data were log-transformed and mean-centered. Data analysis, including multivariate modeling, was performed using R software (version 3.6.3) and SIMCA (version 16.0.2; Sartorius Stedim Data Analytics AB, Umeå, Sweden).

2.8 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol Reagents (Ambion, Austin, TX, USA) and reverse-transcribed with the PrimeScript RT-PCR kit (TaKaRa). Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Premix Ex Taq (TaKaRa) on a LightCycler 480 system (Roche Diagnostics, Germany). Primers sequences (Tsingke Biotechnology, Beijing, China) are detailed in Table S1. Relative mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH serving as the reference gene.

2.9 Statistical analysis

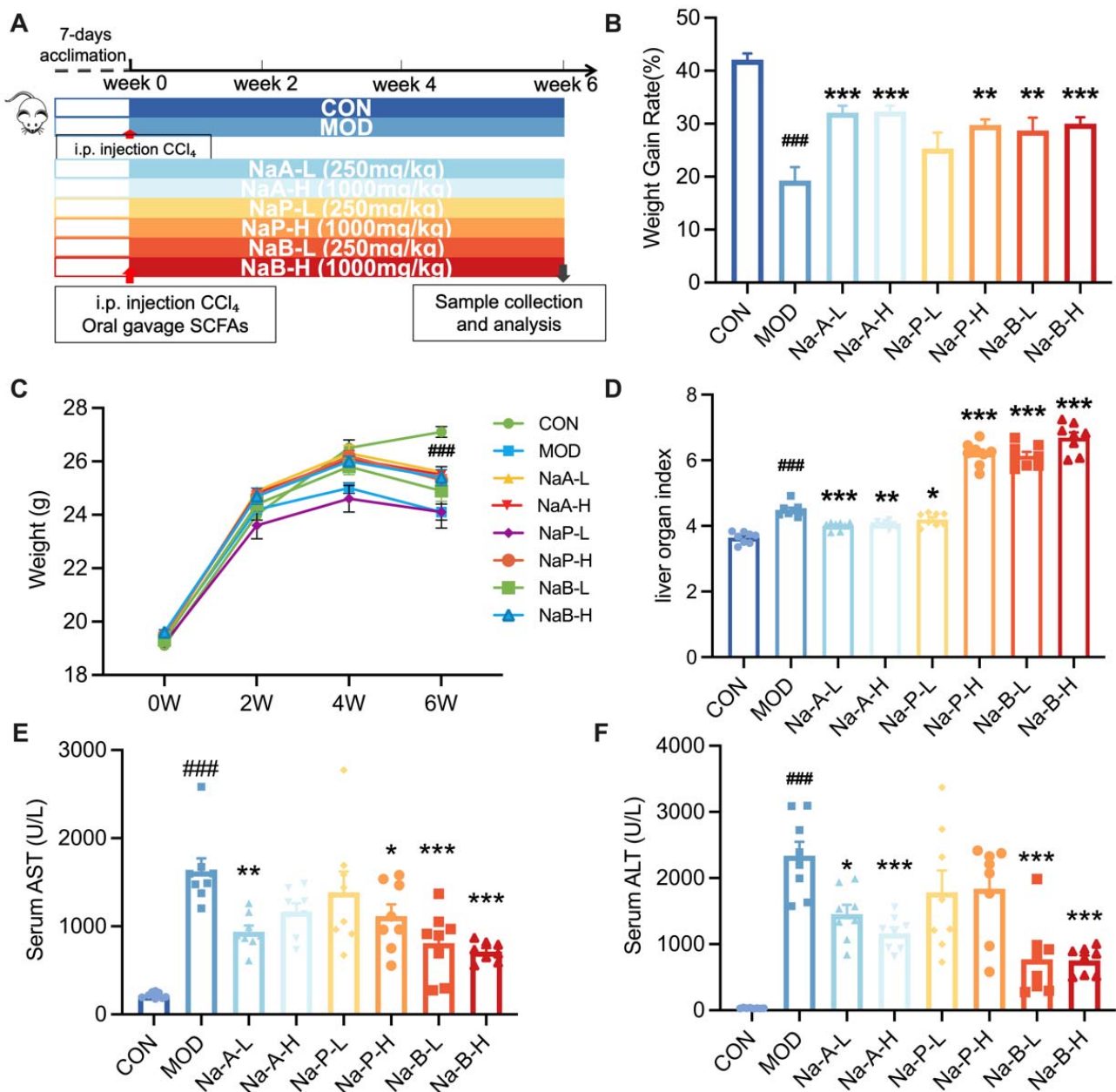
All experiments were presented as the mean $\pm$ the standard error of the mean (SEM). Statistical analysis was conducted using one-way analysis of variance (ANOVA) LSD post hoc tests after normality assessment, using GraphPad Prism version 10.3.1 (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as a two-tailed P -value < 0.05 .

3. Result

3.1 SCFAs ameliorated CCl₄-induced liver dysfunction in mice

Mice injected with CCl₄ were administered three different doses of SCFAs (NaA, NaP, and NaB) via oral gavage to evaluate their protective effects against hepatic dysfunction (Fig. 1A). After six weeks of intervention, mice in the MOD group showed markedly lower weight gain and final body weight relative to controls ($P < 0.001$), accompanied by a higher liver index (Fig. 1B-D). Supplementation with SCFAs,

particularly with NaA and NaB, significantly improved the body weight gain rate (Fig. 1B). Only the low/high-dose sodium acetate (NaA-L/ NaA-H) groups significantly reduced the elevated liver organ index induced by CCl₄. In contrast, neither high-dose sodium propionate (NaP-H) nor low- or high-dose sodium butyrate (NaB-L/NaB-H) produced significant improvements in this metric. Serum levels of ALT and AST, indicative of hepatic injury, were significantly increased in CCl₄-treated mice relative to controls, confirming the successful establishment of liver damage. Compared to the MOD group, NaA and NaB supplementation markedly decreased ALT and AST levels, with NaB-H and NaB-L showing the strongest reductions. Notably, NaA-L produced a marked reduction in ALT and AST, with effects second only to those observed with NaB. NaP exhibited limited efficacy, with only NaP-H modestly reducing AST but having no effect on ALT (Fig. 1E-F). These results suggest that SCFAs administration, particularly NaA or NaB, effectively mitigates CCl₄-induced hepatic dysfunction.



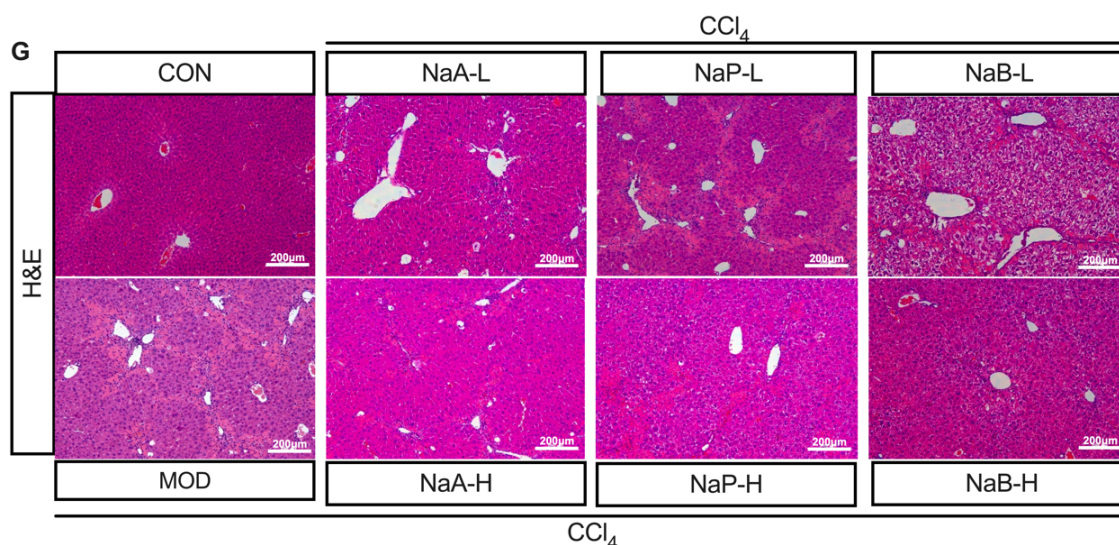


Fig. 1 SCFAs attenuated hepatic injury in CCl_4 -induced mice. **(A)** Animal experimental scheme. **(B)** Body weight gain rate during 6-week gavage treatment in mice. **(C)** Changes in body weight in mice. **(D)** Effect of SCFAs on hepatic organ index. **(E)** Effect of SCFAs on serum activities of AST. **(F)** Effect of SCFAs on serum activities of ALT. **(G)** H&E staining of liver tissue sections (Scale bar, 200 μm). Data are presented as means $\pm$ SEM ($n = 8/\text{group}$). $\#P < 0.05$, $\#\#\#P < 0.001$ vs. CON group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. MOD group. Statistical significance was determined by one-way ANOVA followed by LSD post-hoc tests.

To further elucidate the effects of SCFAs on liver pathology, histopathological analysis was performed. H&E staining revealed significant architectural disruption in the CCl_4 model group, characterized by prominent portal lymphocyte infiltration, focal necrosis, and incomplete bridging fibrosis extending from portal tracts into hepatic lobules. Both NaA-L and NaA-H treatments ameliorated these histopathological changes, preserving hepatic cord integrity and reducing inflammation and fibrosis (Fig. 1G). In contrast, neither dose of NaP improved the structural damage to hepatic tissue. NaB-L exacerbated hepatocellular ballooning degeneration, whereas NaB-H significantly reduced inflammatory infiltration.

Collectively, these results demonstrate that SCFAs, particularly NaA and NaB, exert a multifaceted protective effect against CCl_4 -induced liver injury by improving liver enzyme profiles, ameliorating histopathological damage.

3.2 SCFAs Suppressed HSCs Activation and Attenuate Extracellular Matrix Deposition

Liver fibrosis was evaluated by Sirius Red and Masson's trichrome staining (Fig. 2A-B). The MOD group displayed markedly greater collagen deposition than CON group ($P < 0.001$). SCFA treatment led to a reduction in collagen content, as shown by decreased positive areas in Sirius Red and Masson's trichrome staining. Notably, NaA and NaB exhibited the most pronounced antifibrotic effects, with Sirius Red-positive areas reduced by 32.6% (NaA-L), 38.8% (NaB-L), and 34.9% (NaB-H), and Masson's trichrome-positive areas reduced by 32.2% (NaA-L), 34.5% (NaA-H), and 36.0% (NaP-L), respectively, compared with the model group (Fig. 2A-B).

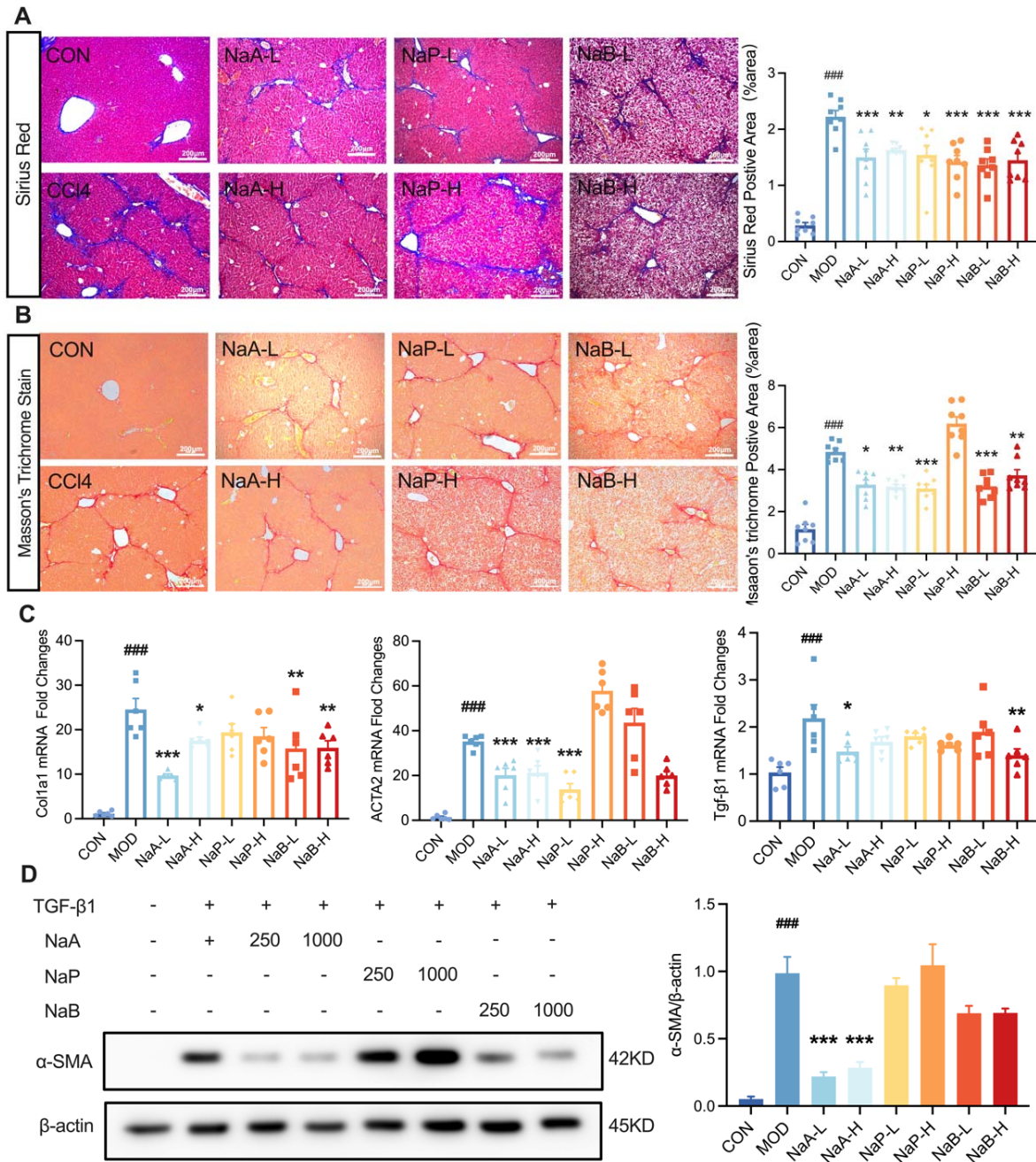


Fig. 2 SCFAs alleviate the activation of hepatic stellate cell and extracellular matrix deposition in CCl₄-induced Mice. (A) Sirius red staining of liver tissue sections (Scale bar, 200 μ m). (B) Masson's trichrome staining of liver tissue sections (Scale bar, 200 μ m). (C) The expression of gene (*Acta2*, *Col1a1*, and *Tgf-β1*) related to tissue fibrosis and collagen deposition were examined using RT-PCR. (D) Western blot image showing α -SMA expression in liver tissues. Data are presented as means $\pm$ SEM (n = 6-8/group). # P < 0.05, ### P < 0.01, #### P < 0.001 vs. CON group; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. MOD group. Statistical significance was determined by one-way ANOVA followed by LSD post-hoc tests.

Expression profiles of key fibrosis-related genes in response to SCFA treatment were also analyzed. NaA-L significantly downregulated *Col1a1*, *Acta2* (α -SMA) and *Tgf-β1* expression (P < 0.05), while other SCFAs treatment groups only exhibited inhibitory effects in individual inflammatory factors. NaA-H, NaB-L, and NaB-H reduced the expression of *Col1a1* by 28.6%, 35.8%, and 35.0%, respectively; NaA-L and NaP-L reduced the expression of *Acta2* by 39.7% and 20.8%, respectively; and NaA-L and NaB-H can reduce the expression of *Tgf-β1* by 32.3% 35.9% (Fig. 2C). Consistent with these findings, Western blot analysis

revealed that only the NaA-L and NaA-H groups exhibited a marked decrease in α -SMA protein expression in liver tissue. ($P < 0.001$) (Fig. 2D).

3.3 SCFAs Attenuated Lipid Deposition and Inflammatory Responses Induced by CCl₄ in mice

Chronic liver injury and oxidative stress contribute to lipid metabolic dysregulation and low-grade inflammation [25]. Oil Red O staining was used to evaluate hepatic lipid accumulation and assess the impact of SCFAs on liver lipid metabolism in CCl₄-treated mice. CCl₄ treatment markedly increased lipid droplet area relative to controls (Fig. 3A). All three SCFAs—NaA, NaP, and NaB—significantly reduced hepatic lipid accumulation, with NaA showing the greatest effect (NaA-H: 67.3% reduction; NaA-L: 63.4% reduction).

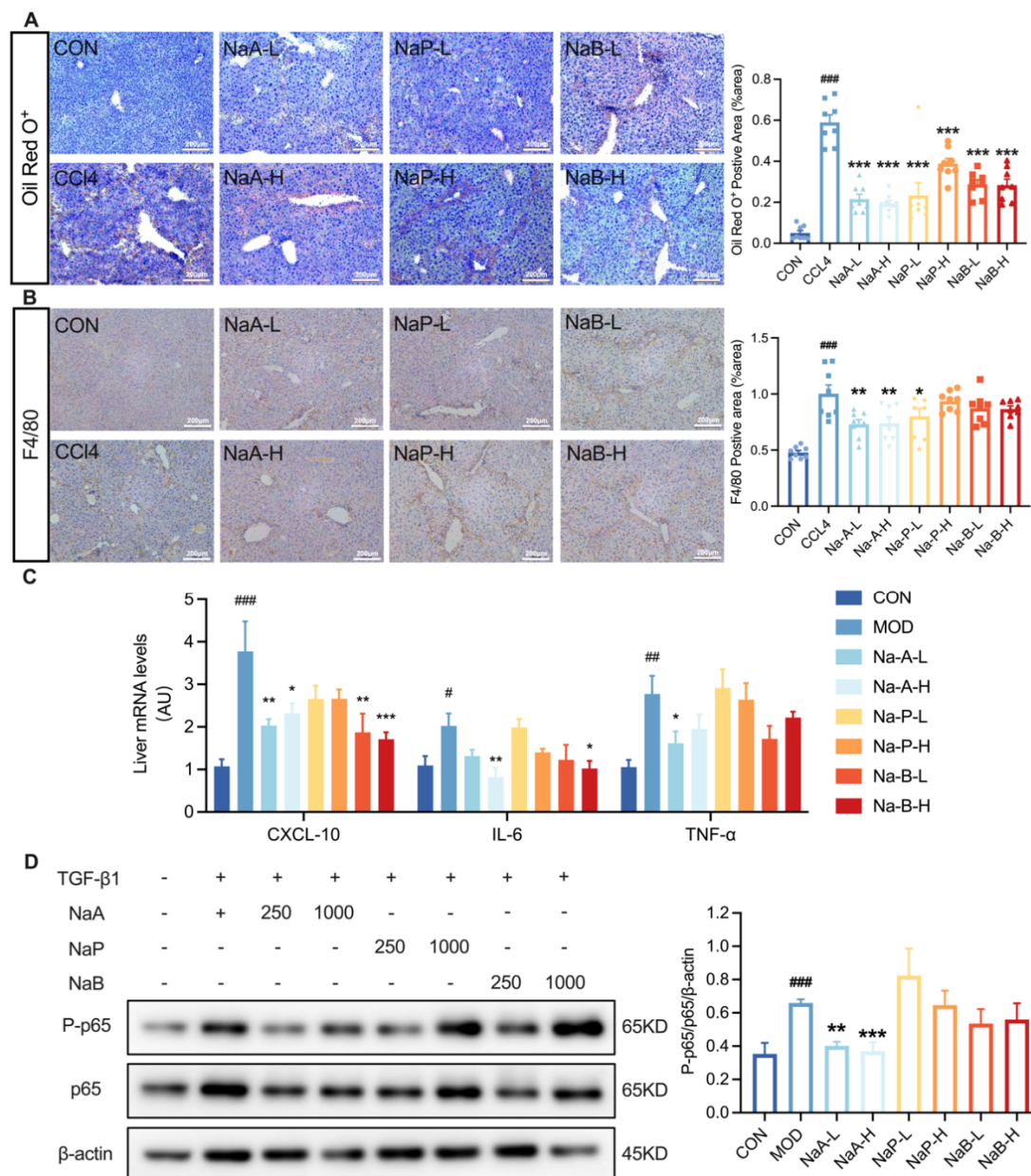


Fig. 3 SCFAs inhibited inflammatory response in liver of CCl₄-induced mice. **(A)** Oil Red O staining of liver tissue sections (Scale bar, 200 μ m). **(B)** IHC staining of F4/80 in hepatic sections (Scale bar, 200 μ m). **(C)** RT-qPCR analysis of inflammatory cytokine genes (*Tnf- α* , *Il-6*, and *Cxcl-10*). **(D)** Western blot analysis of p5 and phosphorylated p5 (p-p65) expression in liver tissue. Data are presented as means $\pm$ SEM ($n = 6$ -8/group). # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CON group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. MOD group. Statistical significance was determined by one-way ANOVA followed by LSD post-hoc tests.

The macrophage infiltration was evaluated by F4/80 immunohistochemistry to assessing the liver inflammation status. Compared to controls, the CCl₄ group showed a 2.1-fold expansion of the F4/80-positive area (**Fig. 3B**). NaA reduced macrophage infiltration, with NaA-H inducing a 67.3% decrease and NaA-L a 27.1% decrease, while NaP treatment yielded a moderate reduction (20.1% decrease).

To elucidate the contribution of pro-inflammatory cytokines to HF, hepatic mRNA expression of C-X-C motif chemokine ligand 10 (*Cxcl-10*), interleukin-6 (*Il-6*) and *Tnf-α* were measured. NaA-L suppressed *Tnf-α* expression, while both NaA-H and NaP-H significantly reduced *Il-6* levels (by 59.6% and 49.4%, respectively). Notably, NaA and NaB consistently downregulated *Cxcl-10* expression across all dosage groups, with reductions ranging from 38% to 55% relative to the MOD group (Fig. 3C). The nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway is a key link in inflammation-driven fibrosis during hepatic fibrosis. Upon activation of NF-κB, inflammatory cells secrete inflammatory factors, which in turn induce the activation of HSCs. Studies have shown that NaA may alleviate liver injury by modulating this pathway. To investigate this mechanism, we used Western blot analysis to assess the ratio of phosphorylated P65 (p-P65) to total P65 in liver tissues. The results indicated that the P-p65/p65 ratio was significantly markedly higher in the MOD group than in the CON group, whereas both NaA-L and NaA-H effectively reduced this ratio and inhibited NF-κB activation (Fig. 3D).

Collectively, NaA, especially low-dose NaA, markedly attenuates key markers of HF, suppresses inflammatory responses, and ameliorates liver injury caused by CCl₄. These data demonstrate the protective effects of NaA on the liver and highlight its potential utility in HF treatment. Further research is needed to elucidate the molecular mechanisms underlying the antifibrotic effects of NaA.

3.4 NaA Inhibited the Activation and Proliferation of LX2 cells

In order to elucidate the molecular pathways involved in NaA-mediated antifibrotic effects, we utilized LX2 cells as an in vitro model. We initially assessed the effects of various concentrations of NaA, alone or in combination with TGF-β1, on LX2 cells viability (Fig. 4A). A clear dose-dependent decrease in cell viability was observed with higher levels of NaA. Cell viability was significantly reduced at NaA concentrations higher than 2 mM ($P < 0.05$) or when cells were treated with 5 ng/mL TGF-β1. Based on these results, we selected 0.1 mM and 1 mM NaA as low and high doses, respectively, for subsequent studies on LX2 cells activation.

Treatment with 0.1 mM or 1 mM NaA effectively inhibited the activation of LX2 cells in response to TGF-β1 stimulation, as evidenced by reduced expression of the ECM marker genes *FN-1* and *COL1A1* ($P < 0.05$) (Fig. 4B). ECM accumulation is also regulated by the equilibrium between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs). Our data showed that both 0.1 mM and 1 mM NaA significantly downregulated *MMP-9* and *TIMP-1* expression, promoting ECM degradation and reducing fibrosis (Fig. 4C). Notably, low doses of NaA are more effective in promoting ECM degradation and reducing the severity of fibrosis. Consistently, Western blot analysis confirmed that both NaA-L and NaA-H significantly reduced α-SMA protein expression ($P < 0.01$), with 0.1 mM NaA exhibiting a more pronounced inhibitory effect. These findings provide additional evidence that NaA suppresses LX2 cell activation (Fig. 4D).

Collectively, these results demonstrate that 0.1mM NaA effectively inhibits LX2 cells activation by regulating ECM production and the expression of matrix-degrading enzymes, thereby providing a molecular basis for its antifibrotic effects.

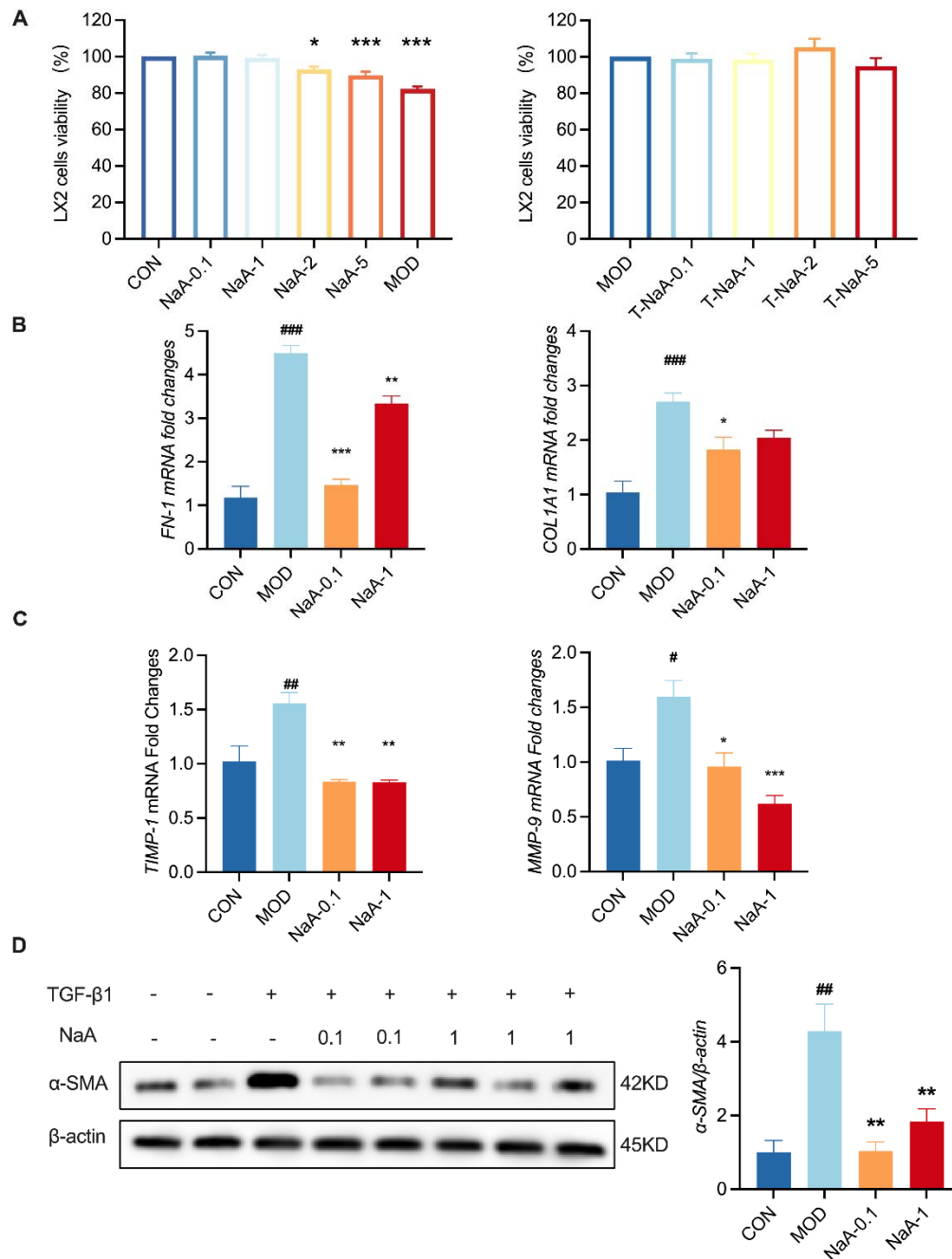


Fig. 4 NaA attenuates fibrotic activation of human LX-2 hepatic stellate cells. (A) Effect of TGF- β 1 (5 ng/mL) and various concentrations of NaA (0.1, 1, 2, 5 mmol/L) on LX2 cells viability.; Effect of TGF- β 1 combinatorial treatment with Sodium Acetate on LX2 Cells Viability. (n=6) (B-C) RT-qPCR analysis of fibrosis- and ECM degradation-related genes (*FN-1*, *COL1A1*, *TIMP-1*, and *MMP-9*) in LX-2 cells. (D) Western blot analysis of α -SMA expression in LX-2 cells. Data are presented as means $\pm$ SEM (n = 3/group). # P < 0.05, ## P < 0.01, ### P < 0.001 vs. CON group; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. MOD group. Statistical significance was determined by one-way ANOVA followed by LSD post-hoc tests.

3.5 Proteomics Reveals Key Targets of NaA in Inhibiting TGF- β 1-Activated LX2 Cells

To elucidate the impact of NaA on TGF- β 1- stimulated LX2 cells, we employed DIA mass spectrometry to comprehensively analyze proteomic alterations in 0.1 mM NaA-treated LX2 cells, with CON and

TGF- β 1-treated models (MOD) as comparisons. DIA analysis identified 6,402 proteins across 9 samples. Principal component analysis (PCA, Fig. 5A) revealed distinct clustering among CON, MOD, and NaA groups, with PC1 and PC2 accounting for 41.8% of the variance. Partial overlap between MOD and NaA groups suggested that NaA partially reversed TGF- β 1-induced proteomic signatures.

Differentially expressed proteins (DEPs) were identified using criteria of fold change > 1.2 or < 0.83 , along with a P -value < 0.05 . The MOD vs. CON comparison showed 712 DEPs (241 upregulated, 471 downregulated), while MOD vs. NaA yielded 205 DEPs (115 upregulated, 90 downregulated; Fig. 5B-C, Fig.S1A). COG/KOG classification of DEPs from MOD vs. NaA highlighted functional categories including posttranslational modification, protein turnover, chaperones, signal transduction mechanisms, cytoskeleton, and general function prediction (Fig. 5D). DEPs between CON and MOD groups are mainly concentrated in post-translational modification, protein turnover, chaperone, and signal transduction mechanisms (Fig.S1B). GO enrichment analysis identified 2,392 terms, including 1,815 biological processes, 279 cellular components, and 298 molecular functions, with enrichment in biological processes such as cellular/protein localization, intracellular organelle, and binding/catalytic activity (Fig. 5E). The GO enrichment bubble plot of CON vs MOD showed that differentially expressed proteins were associated with the cell cycle, cytoskeletal organization, organelle biogenesis, and structural molecular functions (Fig.S1C). KEGG pathway analysis showed that DEPs from the MOD vs. CON comparison were enriched in autophagy, gap junction, and nucleocytoplasmic transport, whereas NaA vs. MOD DEPs were associated with insulin signaling, GnRH signaling, and glycerophospholipid metabolism (Fig. 5F–G, Fig.S1D-E). The protein–protein interaction (PPI) network highlights several key hub proteins, such as ZEB1, RPLP1, EPHB2, KRAS, MAPK8 and LPCAT, characterized by high connectivity and significant expression changes, suggesting pivotal roles in the biological processes analyzed (Fig.S1F).

Intersection analysis identified 25 overlapping DEPs: 10 proteins upregulated by TGF- β 1 and attenuated by NaA, and 15 proteins downregulated by TGF- β 1 but enhanced with NaA. Hierarchical clustering analysis (Fig. 5H) indicated that the expression patterns of these DEPs in the NaA group were similar to those of the control group, but differed markedly from the MOD group, implicating their potential involvement in the antifibrotic mechanism of NaA in LX2 cells. To validate key targets, we focused on the top five DEPs (by $\log_2(\text{FC})$) upregulated by TGF- β 1 and reversed by NaA treatment (Fig.S1G). Based on comprehensive literature review and significance analysis, EPHB2 was identified as a potential central mediator in the antifibrotic effect of NaA. EPHB2 expression was significantly increased in the MOD group, but markedly decreased following NaA treatment ($P < 0.001$) (Fig. 5I). Additionally, another fibrosis marker protein, COL18A1, was also downregulated by 0.1 mM NaA.

In summary, NaA likely ameliorates fibrotic progression in LX2 cells by reversing TGF- β 1-driven proteomic changes, particularly in key proteins such as EPHB2 and COL18A1, and modulating metabolic, cell cycle, and protein synthesis pathways. These observations contribute to a deeper mechanistic insight into the therapeutic potential of NaA for HF.

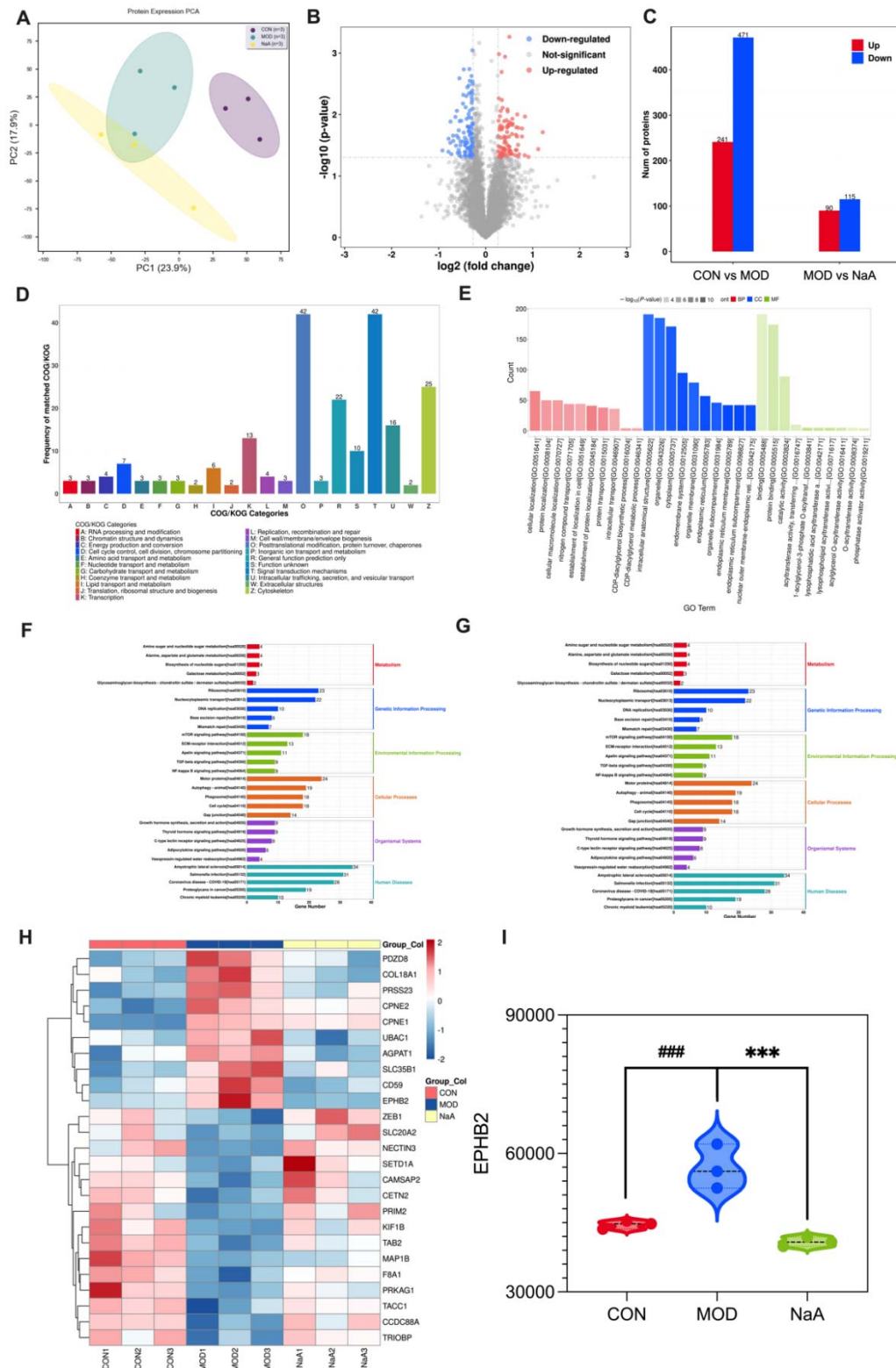


Fig. 5 Proteomic analysis of LX2 cell from the CON, MOD, and NaA groups. (A) PCA of directDIA Proteomics Data: Separation of MOD, CON, and NaA Groups. (B) Volcano plot of differentially expressed proteins: MOD vs. NaA groups. Proteins with fold-change beyond 1.2 or below 0.83 with p value lower than 0.05 were considered as significantly differential expression. Up-regulated proteins (red), down-regulated proteins (blue), and non-significant proteins (grey) are shown. (C) Number of up- and down-regulated proteins in differential proteins in the CON vs MOD group and MOD vs NaA group. (D) COG Functional Classification of Differentially Expressed Proteins in MOD vs. NaA Groups. (E) GO Enrichment Analysis of Differentially Expressed Proteins. KEGG Pathway Enrichment Analysis of Differentially Expressed Proteins in MOD vs. CON Groups (F) and MOD vs. NaA Groups (G). (H) Heatmap of Protein Expression Patterns Across MOD, CON, and NaA Groups. (I) Box plots depicting the relative protein expression levels of EPHB2 in each group. For all bar graphs, data are presented as means $\pm$ SEM (n = 3/group). ###P < 0.001 vs. CON group; ***P < 0.001 vs. MOD group. Statistical significance was determined by one-way ANOVA followed by LSD post-hoc tests.

3.6 NaA Attenuates LX2 Cell Activation by Modulating EPHB2

To validate the proteomic identification of EPHB2 as a potential target of NaA, we examined its expression in LX2 cells stimulated with TGF- β 1. Western blot demonstrated that treatment with varying doses of NaA exhibited a dose effect, with 0.1 mM NaA exerting a more potent reduction in the protein expression levels of EPHB2 and α -SMA (Fig. 6A). In parallel, qPCR analysis demonstrated that *EPHB2* mRNA expression was also significantly reduced, particularly at 0.1 mM NaA (Fig. 6B). These results are consistent with the proteomic findings and provide further evidence that NaA can effectively downregulate EPHB2 expression in activated LX2 cells.

Initially, the effects of various concentrations of the EPHB2 inhibitor AWL-II-49-7 on LX2 cell viability were evaluated using the CCK-8 assay. Results indicated that AWL-II-49-7, at all tested doses, did not significantly influence cell viability. Accordingly, 0.5 μ M was chosen for subsequent experiments to avoid non-specific cytotoxicity. In the context of combination treatment, CCK-8 analysis revealed that NaA concentrations above 1 mM significantly decreased cell viability. Based on these results, we pretreated activated LX2 cells with AWL-II-49-7 (0.5 μ M, 1 hour) and subsequently administered it alone or together with NaA (0.1 mM) for 24 hours (Fig. 6C).

Following these treatments, qPCR analysis (Fig. 6D) revealed that TGF- β 1 stimulation significantly upregulated the mRNA expression of *EPHB2*, *COL1A1* and *FNI*. Treatment with either NaA or AWL-II-49-7 alone significantly downregulated *COL1A1* and *FNI* expression, with AWL-II-49-7 showing a more pronounced inhibitory effect. Notably, the combination treatment did not produce an additive or synergistic suppression of these fibrosis-related genes. Analysis of EPHB2 expression showed that combined NaA and AWL-II-49-7 treatment led to greater suppression of *EPHB2* mRNA than either agent alone. Western blot analysis demonstrated that AWL-II-49-7 or NaA alone significantly suppressed α -SMA protein levels, but their combination did not further enhance this effect (Fig. 6E). These results concluded that EPHB2 acts as an essential downstream mediator in NaA-induced suppression of hepatic stellate cell activation (Fig. 6F).

Collectively, these results indicate that NaA may exert its anti-fibrotic effects, at least in part, by downregulating EPHB2. The enhanced inhibition of EPHB2 observed with the combination therapy suggests that EPHB2 functions as a key downstream effector in NaA-mediated suppression of HSCs activation and fibrosis.

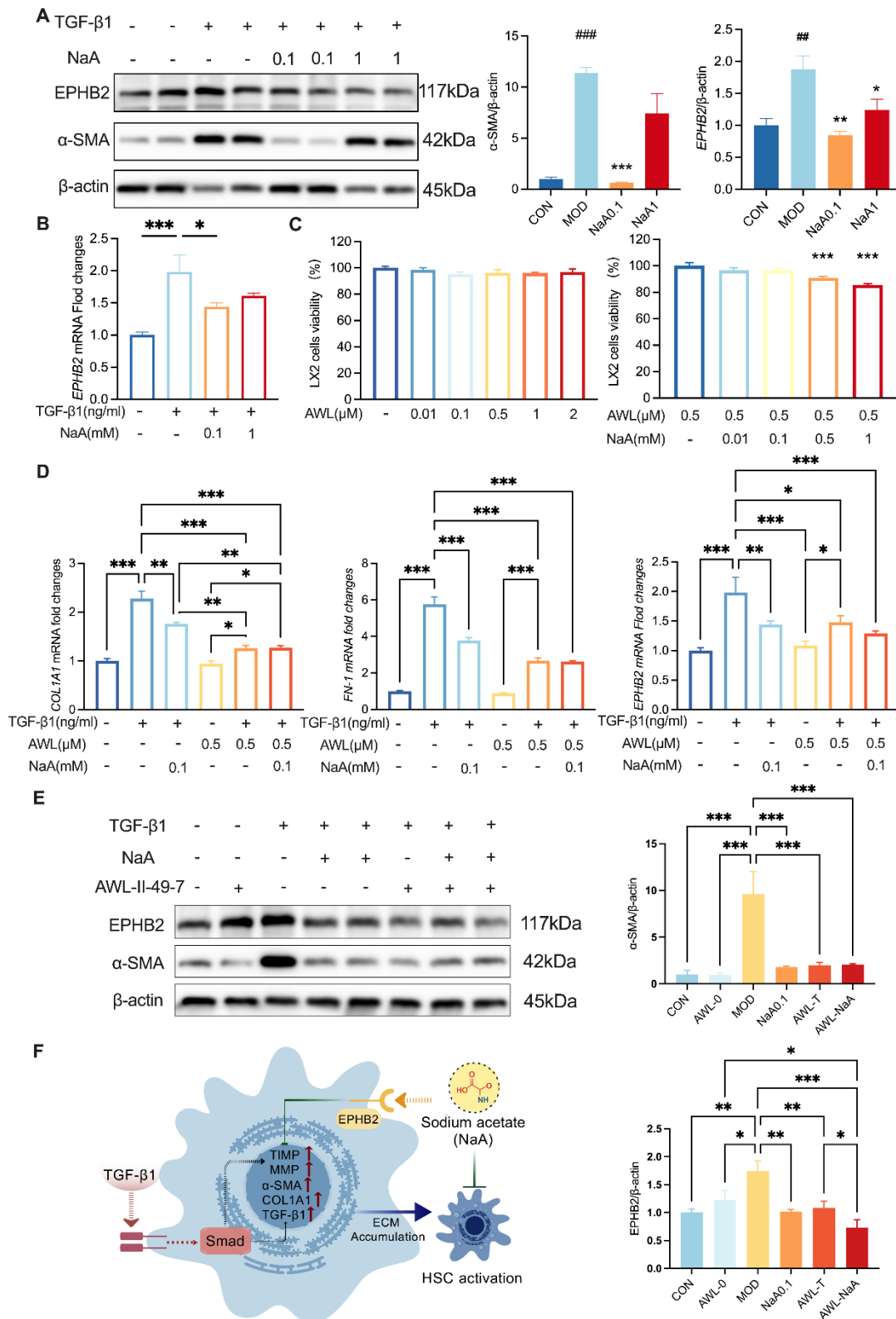


Fig. 6 NaA inhibits TGF- β 1-induced LX2 activation through EPHB2 suppression. (A) Western blot analysis showing the effect of high and low doses of sodium acetate on EPHB2 protein levels in LX-2 cells. (B) RT-qPCR analysis of EPHB2 gene in cells. (C) Effects of different concentrations of AWL-II-49-7 (0.01, 0.1, 0.5, 1, 2 μ mol/L) on LX2 cells viability. Effects of co-treatment with 0.5 μ M AWL-II-49-7 and different doses of sodium acetate on LX2 cells viability. (D) RT-qPCR analysis of *COL1A1*, *FN-1* and *EPHB2* gene in cells. (E) Western blot showing combinatorial effects of AWL-II-49-7 and NaA on EPHB2 and α -SMA suppression. (F) Schematic illustration of the EPHB2-mediated mechanism by which sodium acetate suppresses LX2 cell activation. Created with BioGDP.com [26]. For all bar graphs, data are the mean $\pm$ SEM (n = 3). Data are presented as means $\pm$ SEM (n = 3/group). Statistical significance was determined by one-way ANOVA followed by LSD post-hoc tests for pairwise comparisons between all groups. # P < 0.05, ## P < 0.01, ### P < 0.001 vs. CON group; * P < 0.05, ** P < 0.01, *** P < 0.001 compared between indicated groups.

4. Discussion

This study provides compelling evidence that SCFAs, particularly NaA, exert potent antifibrotic effects by repairing hepatic dysfunction, mitigating hepatic inflammatory injury, and directly modulating HSCs activation. The molecular mechanism of NaA inhibition of HSCs activation was elucidated using a TGF- β 1-induced LX2 cells model in conjunction with proteomics, and it is noteworthy that the anti-fibrotic effect of NaA, as the most potent SCFAs, may be mediated by down-regulation of EPHB2 in LX2 cells. These findings emphasise the therapeutic potential of NaA for treating liver fibrosis and identify EPHB2 as a potential target for antifibrotic treatment.

SCFAs are critical mediators of the gut–liver axis and have gained increasing attention for their immunomodulatory and anti-inflammatory functions. The main SCFAs, including acetate, propionate, and butyrate, significant differences exist in their concentrations, distribution, and biological activity. In the intestine, SCFA levels are highest in the cecum and proximal colon (70–140 mM), declining to 20–70 mM in the distal colon [27]. Once absorbed, their concentrations decrease significantly along the portal circulation: 375 ± 70 $\mu\text{mol/L}$ in the portal vein, 148 ± 42 $\mu\text{mol/L}$ in the liver, and 79 ± 22 $\mu\text{mol/L}$ in peripheral blood, with acetate being the most abundant. In portal blood, the molar ratio of acetate, propionate, and butyrate is roughly 69:23:8, and acetate concentrations are 258 $\mu\text{mol/L}$ (portal), 88 $\mu\text{mol/L}$ (liver), and 29 $\mu\text{mol/L}$ (peripheral) [9]. These distribution patterns are largely due to butyrate's rapid utilization by colonocytes, propionate's hepatic metabolism, and acetate's systemic diffusion [28]. Additionally, tissue-specific expression of SCFAs transporters and receptors (e.g., monocarboxylate transporter (MCT1), GPR41/43) further contributes to their distinct physiological roles. Thus, SCFAs exhibit site-specific effects and dose effects under both normal and pathological states. In this study, the SCFAs exposure levels in both the in vivo and in vitro administration schemes were within the physiological concentration range, slightly higher than in the portal vein and peripheral blood, but far lower than in the intestinal cavity. This is suitable for simulating the normal and pharmacological effects of SCFAs. It is noteworthy that the physiological effects of SCFAs are closely associated with dose. In different studies, the concentration of short chain fatty acids that exert their functions varies, showing significant tissue or disease dependencies [29]. Both the type and dose of SCFAs are crucial for determining their therapeutic potential and physiological impact. Furthermore, a growing body of research highlights the antifibrotic potential of SCFAs in different experimental models. In mouse models of BDL- and schistosomiasis-induced HF, NaB treatment led to marked reductions in serum ALT and AST levels [30,31]. Furthermore, NaA has been reported to alleviate renal fibrosis in unilateral uvula occlusion (UUO) mice by downregulating fibrosis markers, including α -SMA, collagen I, and collagen III [32]. In this study, we evaluated the antifibrotic efficacy of NaA, NaP, and NaB at both low and high concentrations in a CCl₄-induced mouse model of HF. Among the three SCFAs, NaP demonstrated minimal therapeutic benefit, with limited effects on hepatic metabolic function, inflammation, and fibrotic progression. In contrast, high-dose NaB exhibited significant hepatoprotective effects, as evidenced by suppressing hepatic inflammation (*Cxcl-10* and *Il-6*), decreased lipid accumulation and collagen deposition, and downregulation

of fibrosis-related genes such as *Colla1* and *Tgf-β1*. Notably, NaA showed superior efficacy across a broader range of endpoints. Both NaA-L and NaA-H consistently attenuated liver injury and fibrogenesis, with particularly pronounced effects on suppressing fibrosis-related gene expression. These findings suggest that NaA may exhibit a broader therapeutic range and more potent antifibrotic capacity compared to NaP and NaB, warranting further mechanistic investigation.

HSCs are central to the development of HF. Following hepatic injury, HSCs are activated, acquiring myofibroblast-like properties and producing excessive ECM components. Among the myriad profibrogenic signals, TGF-β, primarily released by activated Kupffer cells, injured hepatocytes, and infiltrating immune cells, plays a central role in initiation and maintenance of HSC activation [33]. Stimulation with TGF-β1 upregulates α-SMA and fibrogenic genes such as *Colla1*, thereby amplifying ECM accumulation and promoting fibrosis progression [34]. Activation of HSCs represents the pivotal step in the development of liver fibrosis and is therefore a critical focus for elucidating the mechanisms by which NaA ameliorates fibrotic pathology. To date, there is a lack of direct evidence indicating that SCFAs act specifically on HSCs. In this study, we provide compelling evidence that NaA directly inhibits HSC activation, thereby mediating its antifibrotic efficacy. In vivo, NaA administration markedly ameliorated CCl₄-induced HF, as demonstrated by improved liver histopathology, and significant downregulation of hepatic *ACTA2*, *COL1A1* and *TGF-β1* expression. Consistent with these in vivo findings, in vitro experiments using TGF-β1-stimulated LX2 cells revealed that NaA exhibited a dose effect in inhibiting HSC activation. This suppression was achieved through downregulation of ECM marker genes (*FNI*, *COL1A1*), as well as by promoting ECM degradation via regulation of *MMP-9* and *TIMP-1* levels. These results indicate that NaA attenuates liver fibrosis primarily by directly suppressing HSC activation and modulating ECM turnover.

The mechanisms by which NaA inhibits HSC activation are central to its antifibrotic effects. The activation of HSCs is orchestrated by a complex network of signaling pathways, with TGF-β / Smad [34], PDGF [21], and CTGF [35] being recognized as major drivers. Each of these pathways converges on the transcriptional upregulation of ECM genes such as *COL1A1* and *FNI*, as well as cytoskeletal remodeling through α-SMA induction. While several agents have been shown to interfere with these fibrogenic cascades, such as AMPK agonists [15], HDAC [36], and PPARγ ligands [37], the precise molecular targets and mechanisms for SCFAs, particularly NaA, have remained poorly defined. In the present study, we leveraged DIA-based proteomics to systematically identify molecular changes associated with NaA treatment in TGF-β1-stimulated LX2 cells. This unbiased approach revealed a distinct downregulation of EPHB2, a member of the RTK superfamily, following NaA exposure. EPHB2 exhibits diverse biological activities that are crucial for vascular development, cell adhesion, migration, proliferation, and axon guidance. Recent studies have highlighted the important involvement of EPHB2 in liver diseases. Acting as a downstream effector of PDGF, EPHB2 promotes HSC-driven angiogenesis and tissue remodeling [38]. EPHB2 activation in HSCs further induces the ERK/KLF2/VEGF pathway, facilitating VEGF-mediated migration and recruitment of hepatic sinusoidal endothelial cells [39]. Importantly, it was first demonstrated in 2015 that EPHB2 deficiency in a

CCl₄-induced mouse model resulted in reduced hepatocyte inflammation and markedly attenuated fibrosis [40]. Subsequent studies confirmed that EPHB2 is predominantly expressed and activated in HSCs during CCl₄-induced HF, and that pharmacological inhibition of EPHB2 can ameliorate fibrogenesis [41,42]. In this study, we conducted both combination and single-agent treatments with NaA and the selective EPHB2 inhibitor AWL-II-49-7 in TGF- β 1-activated LX2 cells. Compared to either monotherapy, the combined treatment produced the most pronounced inhibition of EPHB2 expression in LX2 cells. This synergistic effect suggests that NaA may function as an upstream regulator. However, when EPHB2 was inhibited, the antifibrotic effect of NaA was not further enhanced, indicating that the antifibrotic action of NaA is largely dependent on the modulation of EPHB2. Although the precise mechanism by which NaA regulates EPHB2 remains to be elucidated. Enrichment of HDAC2 at the EPHB2 promoter is known to repress its transcription, while increased nuclear acetyl-CoA from pyruvate metabolism can enhance EPHB2 expression via histone acetylation [38,43]. NaA may influence histone acetylation by acting as an HDAC inhibitor or as a precursor for acetyl-CoA [44], thereby regulating *EPHB2* gene transcription. Additionally, SCFA has also been shown to inhibit the activity of pro-inflammatory kinases that crosstalk with EPHB2 signaling. Proinflammatory cytokines such as TNF- α can activate NF- κ B, which in turn binds to the EPHB2 promoter to enhance its transcription [45,46]. NaA has been reported to inhibit NF- κ B signaling in both macrophages and hepatocytes, indicating that downregulation of EPHB2 by NaA may also occur through inhibition of the NF- κ B pathway [15]. Phosphorylation of EPHB2 is also subject to MAPK pathway-mediated phosphorylation of ERK1/2 to inhibit EPHB2 kinase activity in fibrosis [47].

Despite these insights, our study has certain limitations. The detailed molecular mechanisms by which NaA interacts with its target EPHB2 require further elucidation. Future research using knockout animal models will be important to validate the causal role of EPHB2, while targeted delivery strategies may enhance hepatic specificity and therapeutic efficacy. Additionally, clinical studies are warranted to evaluate the relevance and translational applicability of SCFA-based interventions in human liver fibrosis.

5. Conclusions

In this study, we provide the first evidence that low-dose NaA exerts the most pronounced inhibitory effect on CCl₄-induced HF. We found that NaA acts primarily by directly suppressing the activation of HSCs, the principal effector cells in hepatic fibrogenesis. Through DIA-based proteomics combined with validation using a specific inhibitor, EPHB2 was identified as a novel downstream target mediating this antifibrotic effect. These results not only illuminate the pathways through which SCFAs inhibit fibrosis, but also informs the development of precise and safe therapeutic approaches for chronic liver disease.

Ethics statement

Animal Research approved by the Ethics Committee of China Agricultural University (Approval number: Aw90801202-4-1).

Declaration of competing interest

The authors 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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Data availability

Data will be made available on request.

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