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Decreased PPAR β in hepatocytes specifically determines BPA-induced selective accumulation of liver glycerolipid and cholesterol esters

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ABSTRACT: The rapidly increasing prevalence of metabolic dysfunction-associated steatotic liver disease (MASLD) is driven by endogenous risk factors. Bisphenol A (BPA), an endocrine disruptor chemical commonly found in food packaging, although it has been clearly defined as a typical risk factor for MASLD, the lipid-specific and cell-specific mechanisms leading to hepatic steatosis remain unclear. Our results showed that BPA exposure induced significant hepatic steatosis and dyslipidemia in C57BL/6 male mice. Furthermore, lipid composition analysis revealed that BPA induced specific accumulation of cholesteryl esters (CE 20:6 and CE 19:0) and glycerolipid subclasses (TAG and DG), rather than phospholipids. Furthermore, decreased peroxisome proliferator-activated receptor β (PPAR β) results in over-synthesis of cholesterol and glycerolipid, and reduced fatty acid oxidation. Overexpression of PPAR β alleviated BPA-induced lipid dysregulation by disrupting the INSIG/SREBP pathway, a critical regulator of glycerolipid and cholesterol esters synthesis. Besides, BPA-induced hepatic steatosis presents significant cell-type specificity. The abnormal expression of PPAR β and lipid accumulation were only observed in hepatocytes rather than non-parenchymal cells. More deeply, PPAR β overexpression in hepatocytes attenuated BPA-induced steatosis, whereas its knockdown exacerbated lipid dysregulation, underscoring its essential and cell-type-specific role in maintaining hepatic lipid homeostasis. These findings provide important insights into the mechanisms of hepatic lipid dysregulation caused by BPA and highlight the protective effect of PPAR β in lipid metabolism. This provides a crucial theoretical foundation for preventing liver diseases associated with contaminants in food packaging.

Keywords: Bisphenol A, PPAR β , Hepatocytes, Glycerolipid synthesis, Cholesterol synthesis.

1. Introduction

As the most common liver diseases worldwide, MASLD affects approximately 32.4% of the global population, and its prevalence is increasing annually^[1]. Moreover, the disease is occurring at a younger age^[2]. Epidemiological studies have demonstrated that exposure to environmental risk factors during early life, such as heavy metals, environmental endocrine disruptors (EDCs)^[3-5], and organic pollutants, can increase susceptibility to metabolism-related diseases^[6, 7]. Bisphenol A (BPA) is a lipophilic chemical and can cause enrichment of organisms through bioconcentration and amplification in food chains^[8, 9]. Long-term exposure

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to low doses of BPA always has a delayed and detrimental effect^[10], which means that when the exposure occurs during the embryonic^[11, 12], fetal or neonatal period, the damage does not manifest itself until the offspring matures^[13, 14]. As a result, the damage is inconspicuous. Therefore, it is important to identify key genes that may systemically ameliorate the metabolic diseases.

BPA has been detected in blood, urine, placenta, breast milk, amniotic fluid, and adipose tissue or placenta tissues of mammalian^[15, 16]. Population-based cohort studies have demonstrated that prenatal exposure to BPA is linked to an increased risk of liver injury and elevated levels of apoptotic hepatocytes, indicating that it may be a potential risk factor for hepatic lipid metabolism^[17]. Similarly, several previous studies have demonstrated that BPA can impact liver injury by promoting lipid accumulation. For instance, BPA exposure has induced the increased appetite and hepatic steatosis through activation of the zebrafish cannabinoid receptor type 1 (CB1)^[18, 19]. Covalent protein modification has been shown to be an unignorable factor in BPA hepatotoxicity^[20]. Moreover, low-dose prenatal BPA exposure has interfered with hepatic metabolic pathways and gut microbiota in mice^[21]. However, the potential toxicity mechanisms of BPA on MASLD have remained to be unclear. Hence, further exploration is required to understand the underlying mechanisms of hepatic steatosis caused by BPA exposure.

The pathogenesis of MASLD entails dynamic alterations and functional disturbances across multiple lipid species. Although triglycerides (TGs) accumulate substantially during hepatic steatosis, they may function primarily as buffers against lipotoxic effects rather than direct mediators of injury^[22]. Conversely, a decreased phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio, aberrant cholesteryl ester accumulation, and the pro-apoptotic effects of ceramides (CER) collectively form a multi-layered pathogenic network of lipids in MASLD^[23, 24]. BPA exposure markedly elevates hepatic levels of triglycerides and total cholesterol, promotes lipogenesis by activating lipidogenic genes such as SREBP1, ACC1, and FAS, and enhances the accumulation of sphingolipids (e.g., hexosylceramide) and diacylglycerol (DAG)^[25]. These changes disrupt membrane integrity, induce insulin resistance, and activate inflammatory pathways including TLR4/NF- κ b^[26].

MASLD involve functional dysregulation and disrupted intercellular communication among multiple hepatic cell types under physiological and pathological conditions^[27]. In the healthy liver, hepatocytes maintain lipid metabolic homeostasis^[28]; Kupffer cells (KCs) contribute to immune surveillance and clearance of apoptotic cells^[27]; hepatic stellate cells (HSCs) remain quiescent and store vitamin A^[29]; and endothelial cells regulate sinusoid permeability and immune cell trafficking^[30]; Furthermore, BPA exposure has been shown to perturb the function of multiple hepatic cell types. However, the cell-type-specific mechanisms through which BPA contributes to MASLD development remain poorly understood, particularly regarding intercellular crosstalk and phenotype-specific effects.

Hepatic steatosis is the primary pathogenic feature of MASLD^[31]. As known, the reprogramming of genes and proteins related to regulate lipid homeostasis. In our previous study, BPA exposure induced lipid droplet formation and peroxisome proliferator-activated receptor (PPAR) pathway played a crucial role in the

process^[32]. PPARs, including PPAR α , PPAR δ and PPAR γ , are ligand-activated transcription factors of nuclear hormone receptor superfamily. As known, PPARs act as core lipid sensors involved in the regulation of lipid homeostasis. Several studies have demonstrated that BPA exposure disrupted hepatic lipid metabolism by suppressing PPAR α in the liver and promoting the expression of PPAR γ protein in adipose tissue^[33,34]. However, there was limited information available on the role of PPAR β in liver-related diseases caused by BPA exposure.

PPAR β is a well-established regulator of lipid metabolism and its downstream target molecules are closely associated with fatty acid oxidation (FAO) and lipid synthesis. A previous study has demonstrated that the overexpression of PPAR β in the liver enhances AMPK phosphorylation, thereby promoting the FAO process^[35]. Interestingly, PPAR β is involved in the transcriptional regulation of insulin-induced genes (*INSIGs*), which have PPAR response elements (PPRE) in promoter regions^[36]. *INSIGs* inhibits the activation of sterol regulatory element-binding protein 1/2 (SREBP1/2) by binding to SREBP cleavage activating protein (SCAP), thereby inhibiting its cleavage to SREBP1/2^[37]. SREBP1/2 is critical for lipid metabolism, with SREBP1 regulating the transcription of lipid synthesis-related molecules and SREBP2 involved in the regulation of cholesterol synthesis^[37]. Studies have shown that BPA promotes the expression of SREBP1 and its pro-lipogenic related enzyme, which can lead to hepatic steatosis^[38]. Thus, PPAR β may play important roles in the development of the hepatic lipid metabolism disorders caused by BPA exposure.

Here, we combine rodent models, cell-type-specific analyses, and multi-omics approaches to: (1) identify hepatocytes as the primary target of BPA-induced PPAR β suppression and the consequent accumulation of specific lipid species including cholesteryl esters and sphingolipids; (2) delineate the PPAR β -*INSIG*/SREBP axis as the core pathogenic pathway driving hepatic lipid metabolic dysregulation; and (3) demonstrate complete metabolic rescue via hepatocyte-specific PPAR β overexpression. Our work establishes PPAR β as both a biomarker and therapeutic target for environmental metabolic disease. These findings provide new insights into the molecular mechanisms behind BPA-induced liver damage.

2. Materials and methods

2.1 Animals and treatments

Eight-week-old C57BL/6 mice were obtained from Jiangsu Huachuang Xinnuo Pharmaceutical Science and Technology Co. and housed in the Specific Pathogen Free (SPF) grade animal facility of Hefei University of Technology. After one week of acclimatization, mice were mated in a 2:1 ratio of males (n=20) to females (n=40). Female mice with vaginal plugs were designated as days of gestation (GD0) and housed individually. All pregnant mice were divided randomly into two groups. One group of pregnant mice was gavaged with a 500 μ g/kg dose of BPA per day from GD0 (BPA group) until postnatal day 21 (PND21), while the Ctrl group received ultrapure water containing 2% DMSO (Ctrl group). Male offspring from both groups were similarly exposed to either BPA or the DMSO solution from PND21 until 16 weeks postnatally. Mice in the experimental group were exposed to a daily dose of BPA at 500 μ g/kg from pregnancy until 16 weeks postnatally. The animals were kept in the following conditions: temperature 18°C-22°C, humidity 40%-60%,

light cycle of 12 h light/12 h dark, and free feeding and drinking during the experiment. Meanwhile, the animal experiments were approved by the Animal Ethics Committee of Hefei University of Technology (HFUT20240227003).

2.2 Hematoxylin & eosin (H&E) and Oil Red O staining

Mouse liver and epididymal adipose tissues (EAT) were fixed in 4% paraformaldehyde, and 15 μ m thick sections were prepared through paraffin embedding. The tissues were stained with hematoxylin for 5 min and then rinsed in water for 1 h. Subsequently, the sections were stained with eosin for 2 min, dehydrated, hyalinized, and sealed. For liver tissues, sections were cut at 15 μ m using a cryostat after fixation in 4% paraformaldehyde. These sections were treated with 75% ethanol for 1 min, stained with oil red O dye for 15 min, washed to remove excess dye, and then stained with hematoxylin for 2 min before sealing. Tissue photomicrographs were captured using a Pathology Slide Scanner (SLIDEVIEW VS200, Olympus, Tokyo, Japan).

2.3 Biochemical parameter determination

Serum samples from mice were collected by centrifugation at 3000 rpm for 20 minutes. Biochemical parameters, including triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), alanine transaminase (ALT), and aspartate aminotransferase (AST), were assessed using an automated biochemical analyzer (Chemray 800, Shenzhen, China).

2.4 Hepatic TG measurement

The liver tissues were homogenised using a phosphate buffer as the solvent. Samples, standards, and colour development reagents were added sequentially and incubated at 37 °C for 10 min. To measure the concentration of TG in the samples, the kit's specifications were followed. The absorbance value at 500 nm was measured to obtain the concentration of TG in the samples.

2.5 RNA expression assay

Total RNA was extracted from liver tissues using the QIAzol Lysis Reagent (Qiagen, Germany). mRNA was then fragmented to approximately 300 bp and cDNA was synthesized with reverse transcriptase (Invitrogen, CA, USA), using the mRNA as a template. After quantification with a Qubit 4.0, a paired-end RNA-seq library was sequenced on a NovaSeq 6000 sequencer (2×150 bp read length). Differentially expressed genes (DEGs) between the Ctrl and BPA groups were identified using the R package. Genes with an absolute log₂ fold-change ($|\log_2FC|$) of ≥ 1 and an adjusted p-value (FDR) of < 0.05 were considered significantly differentially expressed. KEGG pathway analysis was performed using KOBAS. Additionally, RNA extraction was also conducted with TRIzol® Reagent (Qiagen, Germany) following the manufacturer's instructions. The raw RNA-sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under the Submission ID: SUB15919464.

2.6 Preparation of protein samples and western blots

The samples were homogenised in a protein lysate containing 1% protease inhibitor (TIANGEN, China). The lysate was then centrifuged at 12,000 rpm at 4 °C. Protein extracts were denatured by heating in boiling water for 15 min. Then, the proteins were separated by SDS-PAGE and electrophoretically transferred onto a PVDF membrane (Millipore, MA, USA) under the condition of 350 mA for 100 min. The PVDF membrane was soaked with skimmed milk to block the non-specific binding sites. The PVDF membrane was then sequentially incubated with specific primary and secondary antibodies. Immunoblot signals were detected using ECL Western blotting detection reagent (TransGen, Beijing, China) and bands were quantified using Image J software (Bio-Rad, Harkles, CA, USA). Primary anti-bodies used were as follows: GAPDH (Proteintech, Cat No. 60004-1-Ig, 1:1000, 36 kDa), PPAR α (Proteintech, Cat No.66826-1-Ig, 53kDa), PPAR β (Proteintech, Cat No.60193-1-Ig, 54 kDa), PPAR γ (Proteintech, Cat No.16643-1-AP, 58 kDa), Insulin induced gene 1(INSIG1) (Proteintech, Cat No.55282-1-AP, 35 kDa), Insulin induced gene 2(INSIG2) (Proteintech, Cat No.24766-1-AP, 30 kDa), Sterol regulatory element-binding transcription factor 1(SREBP1) (Proteintech, Cat No.14088-1-AP, 125), Sterol regulatory element-binding transcription factor 2(SREBP2) (Proteintech, Cat No.28212-1-AP, 120 kDa), 3-hydroxy-3- methylglutaryl-Coenzyme A reductase (HMGCR) (Proteintech, Cat No.13533-1-AP, 95 kDa), Squalene epoxidase(SQLE) (Proteintech, Cat No. 12544-1-AP, 50 kDa), Fatty acid synthase(FASN) (Proteintech, Cat No : 10624-2-AP, 272 kDa).

2.7 Plasmid construction

To achieve overexpression of PPAR β in cells, the PPAR β cDNA (NM_001411509) was amplified and cloned into empty vectors (GL180) using the following primers: 5'-CCGGGCTGTAATTAGCTGAG and 5'-GACACGCTGAACTTGTGGC. The resulting plasmid vector was packaged into lentivirus (pcSLenti-EF1-EGFP-P2A-Puro-CMV-Ppdr-3xFLAG-WPRE) and utilized for subsequent experiments after titration. For PPAR β RNA interference, the targeting fragment GATCCGCAAGATCCAGAAGAAGAACCTTCAAGAGAGGTTCTTCTTCTTCTGGATCTTGCTTTTT TTTA was inserted into the empty pRNAT-U6.1/neo vector (Promega, Madison, WI, USA).

2.8 Adeno-associated virus (AAV) delivery

The modified vector was utilized to insert the cDNA sequence of PPAR β (NM_001411509) into the GL3051 vector (pAAV-CBh-EGFP-WPRE) for PPAR β expression. Adeno-associated virus (AAV) vectors, specifically AAV2/8-CBh-Ppdr-3xFLAG-P2A-EGFP-WPRE and AAV2/8-CBh-EGFP-WPRE (Ctrl), were designed and provided by Obio Technology (Shanghai, China). Subsequently, AAV2/8 viruses expressing PPAR β were injected into the liver of mice via tail-vein injection, using 1×10^{11} viral genome copies diluted in 50 μ L of warm sterile saline per mouse. After a treatment period of three months, the mice were sacrificed for quantitative RT-PCR analysis to confirm PPAR β expression and function.

2.9 Cell culture and treatment

Murine hepatocellular carcinoma cells, Hepa1-6, and murine AML12 cells were sourced from the Cell Repository of the Chinese Academy of Sciences. They were cultured in DMEM medium supplemented with

5% fetal bovine serum (Gibco, Waltham, MA, USA) in a humidified environment at 37°C with 5% CO₂. For experimental treatment, the cells were exposed to 10 µM BPA for 48 hours.

2.10 LCMS analysis of lipids

The tissue sample preparation involved homogenizing the tissue at a 1:4 mass-to-volume ratio using a methanol solution containing internal standards (ketoprofen at 0.4 µg/mL and 2-chloro-L-phenylalanine at 1.0 µg/mL). After adding 1 mL of MTBE and vortexing for 15 min, 250 µL of MS-grade water was added, followed by incubation at room temperature and centrifugation at 14000 g for 15 min. The organic phase was collected, evaporated, reconstituted with methanol-acetonitrile (1:1), vortexed, centrifuged, and the supernatant was transferred for analysis.

Untargeted Lipidomics analysis was performed on an Agilent 1260 infinity II Bio-inert LC system coupling to Agilent 6230 Q-TOF MS (SG22180102) with infinityLab poroshell 120 EC-C18 column (4.6×100 mm, 2.7 µm) at 32 °C. The flow rate was 0.3 mL/min and the injection volume was 3 µL. The scan range was 50-1500 m/z. Dual ESI source was used with positive and negative ion modes. The gas temperature was set at 350°C. Fragmentor was 120 V. The flow rate of drying gas was 11L/min. To provide more reliable experimental results during instrument testing, the samples are randomly ordered to reduce system errors. QC samples were interspersed throughout the analytical run at a frequency of one every 10 injections.

The mass spectrometry raw data collected by LC-MS was imported into Agilent MassHunter Profinder B.06.00 for data processing, this mainly included peak extraction, peak alignment, and compound identification. Metaboanalyst platform (www.metaboanalyst.ca) was used for data preprocessing, statistical analysis, metabolite classification labeling, and functional labeling. The multivariate raw data was dimensionally reduced by principal component analysis (PCA) to analyze the groupings, trends (intra-group and inter-group similarities and differences) and outliers of the observed variables in the data set (whether there is an abnormal sample). Using Partial Least Squares Method-Discriminant Analysis (PLS-DA), the Variable Importance in Projection (VIP), the variability analysis, the fold change and the student's t test to screen for differential metabolites. During the analysis, the data were log₂ transformed and z-score normalized. The clustering algorithm used hierarchical cluster, and the distance calculation was performed in Euclidean distance. Metabolic pathway enrichment analysis of differential metabolites was performed based on the KEGG database. Metabolic pathways with $P < 0.05$ were significant enriched by differential metabolites. The raw metabolomics data have been deposited in the Metabolomics Workbench under the Project ID: DataTrackID6963.

2.11 Immunofluorescence

Liver tissue sections were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.1% Triton X-100 for 10 min. After washing with PBS, the sections were blocked with 5% bovine serum albumin (BSA) for 1 hour at room temperature. Primary antibodies targeting PPARβ (Proteintech, Cat No.60193-1-Ig, 54 kDa, 1:200 dilution) were applied and incubated overnight at 4 °C. After washing, tissue sections were incubated with fluorophore-conjugated secondary antibodies (1:500

dilution) for 1 hour in the dark. Nuclei were counterstained with DAPI for 5 min. Images were captured using a confocal microscope (model), and quantification of fluorescence intensity was performed using ImageJ software. All imaging was carried out under consistent exposure conditions across groups.

2.12 Lipid Fluorescence Staining

Preparation of staining solution: Dissolve BODIPY 493/503 (GlpBio, USA) in DMSO to create a stock solution at 10 mM. Dilute the stock solution in PBS to prepare a working solution at 10 μ M. Cell staining: Plate adherent cells on sterile coverslips. After removing the coverslip from the medium, add 100 μ L of the working solution to one corner, ensuring even coverage of the cells. Incubate at 37°C, protected from light, for 5-30 min. Microscopy detection: BODIPY 493/503 has excitation/emission maxima at 493/503 nm for imaging.

2.13 Extraction of primary mouse liver cells.

Anesthesia was administered to 8-week-old male C57 mice. Upon dissection, the abdominal and thoracic cavities were exposed. The suprahepatic segment of the inferior vena cava was ligated, and the needle was inserted at the level of the inferior vena cava. Perfusion was initiated, and a small incision was made at the portal vein. The liver gradually swelled, and gentle pressure with a glass dissector caused indentation with slow recovery, indicating completion of the procedure. For isolation, the liver was immersed in a glass dish containing 3 mL of 10% fetal bovine serum (FBS) to terminate perfusion, followed by the addition of 10 mL of washing solution. Fibrous bundles were grasped with forceps, and the liver was shaken to dislodge residual cells. The cell suspension was filtered to obtain a hepatocyte suspension. Purification was performed by transferring the filtered hepatocyte suspension into a 50 mL centrifuge tube and centrifuging under cold conditions (4 °C, 50×g, 3 min). The supernatant was discarded, and 15 mL of washing solution was added, followed by three gentle pipetting steps. This procedure was repeated three times. The supernatant was discarded again, and the cells were resuspended in 10 mL of complete culture medium. The cell pellet was suspended in PBS and subjected to cell separation via a linear Percoll gradient.

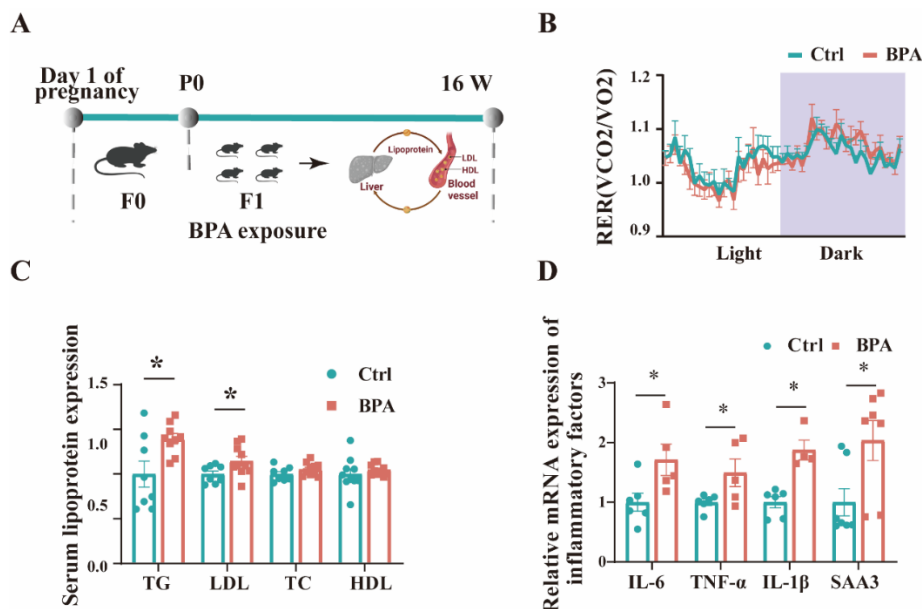
2.14 Statistical Analysis

All data were plotted in figures as mean $\pm$ SEM. Data analysis was performed with GraphPad Prism (version 8.0.2). Statistical differences were analyzed by unpaired T test, one-way ANOVA followed by Fisher's LSD post-hoc test. For a more detailed explanation, see the accompanying figure legends. Unpaired T test or one-way ANOVA followed by Fisher's LSD post-hoc test was used to calculate the significance of a difference between two or more groups, respectively. The statistical tests were two-sided, and the significance levels were defined as follows: * Represents the significance at $P < 0.05$. ** Represents the significance at $P < 0.01$. *** Represents the significance at $P < 0.001$. NS represents the significance at $P > 0.05$.

3. Results

3.1 Exposure to BPA causes dyslipidemia and promotes hepatic steatosis.

To investigate the metabolic impacts of BPA, we administered BPA (or vehicle control) to male C57BL/6 mice via oral gavage for 8 weeks (Fig. 1A). Throughout the exposure period, food intake, body weight gain, fat mass, and the respiratory exchange ratio (oxygen and carbon dioxide exchange) were monitored. No significant differences were observed in these parameters between the BPA-treated and Ctrl groups (Figure 1B S1A-C S2A-B). Serum lipid concentrations were measured, and we found that triglyceride (TG) and low-density lipoprotein (LDL) levels were significantly elevated in the BPA group. However, total cholesterol (TC) and high-density lipoprotein (HDL) levels remained unchanged (Fig 1C). Additionally, serum levels of glucose (GLU) and glycated serum protein (GSP), markers of glucose metabolism homeostasis, were unaffected in the BPA group (Fig S2C-D). Similarly, glucose tolerance tests showed no significant differences in blood glucose levels between BPA-exposed mice and the Ctrl group (Fig. S2E-F). The experimental results indicate that BPA exposure disrupted lipid metabolism in mice. Given the critical roles of adipose tissue and the liver in lipid metabolism, we examined the effects of BPA on both organs. BPA exposure was found to significantly increase the expression of pro-inflammatory genes in the liver (Fig 1D). H&E staining revealed a significant increase in adipocyte diameter (Fig 1E) and (Fig S2G). H&E staining revealed numerous fatty vacuoles in the hepatic tissues of the BPA group (Fig 1F), while Oil red O staining showed a greater number of lipid droplets compared to the Ctrl group (Fig 1G) and (Fig S2H). Additionally, BPA exposure significantly increased the liver weight coefficient (Fig 1H). Furthermore, serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated in the BPA-exposed group (Fig 1I-J). However, compared with the Ctrl group, female offspring exposed to BPA showed no significant differences in key metabolic parameters, including blood lipid profiles, liver weight and adipose tissue mass. This lack of effect in females contrasts sharply with the pronounced metabolic dysregulation reported in males, which strengthens the evidence for a sex-specific effect of BPA on developmental metabolic toxicity (Fig. S3). Thus, we demonstrated that BPA exposure affects lipid metabolism in male C57 mice, particularly impairing liver function and leading to the development of MASLD.



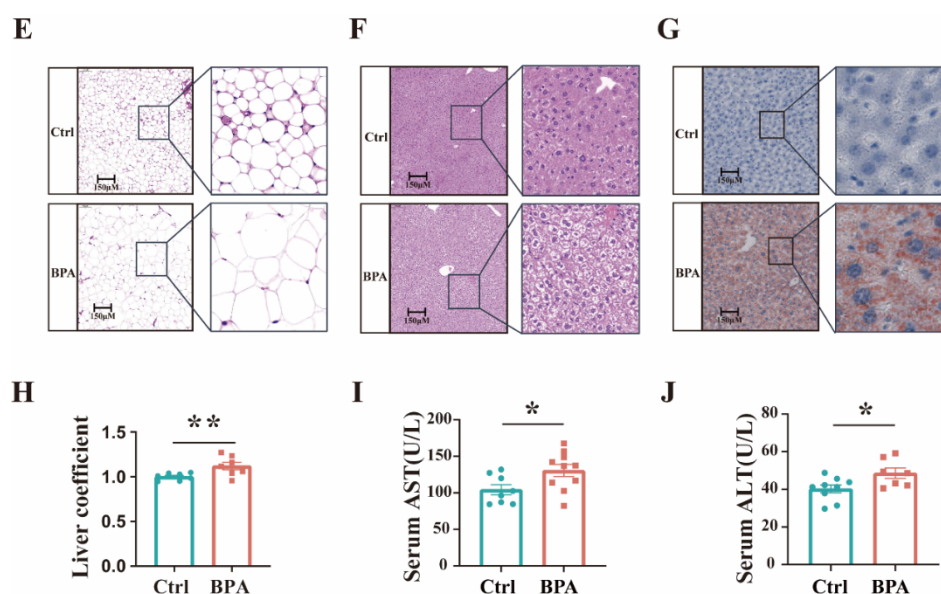


Fig. 1. BPA exposure disrupts lipid homeostasis and induces MASLD in mice.

Male offspring were continuously exposed to BPA from the first day of gestation until 16 weeks postpartum. (A) Drug exposure regimens in mice. (B) Gas exchange ratios of oxygen consumption and carbon dioxide production in mice, measured using metabolic cages (n=12 per group). (C) Serum levels of triglycerides (TG), low density lipoprotein (LDL), total cholesterol (TC) and high density lipoprotein (HDL) (n=8 per group). (D) Expression of inflammation-related genes in the liver of mice (n=5 per group). (E F) Representative micrographs of epididymal fat and liver H&E staining (n=3 per group).

(G) Representative micrograph of liver Oil Red O Stain (n=3 per group). (H) Liver mass coefficient for mice (liver mass/body weight). (I J) Serum levels of liver injury-related transaminases ALT and AST (n = 8 per group). Note: For quantitative analysis of micrographs, the ImageJ software (Bio-Rad, Hercules, CA, USA) was utilized. All data were plotted in figures as mean ± SEM. Statistical differences were analyzed by unpaired T test. The statistical tests were two-sided, and the significance levels were defined as follows: * Represents the significance at $P < 0.05$. ** Represents the significance at $P < 0.01$. *** Represents the significance at $P < 0.001$. NS represents the significance at $P > 0.05$.

3.2 BPA exposure disrupts cholesterol and triglyceride homeostasis in the liver

We have confirmed that BPA exposure induces lipid abnormalities in the liver. However, the specific lipid species involved remain unclear. To address this, we performed lipidomics and metabolomics to measure the changes in hepatic lipid species under BPA exposure. The LC/MS analysis revealed distinct ion peak profiles in both positive and negative ion modes, highlighting metabolite variations between BPA and Ctrl group (Fig 2A). Principal Component Analysis (PCA) further demonstrated clear separation between these groups, indicating significant differences in overall metabolite composition (Fig 2B). The volcano plot identified numerous differentially expressed metabolites in the BPA group, suggesting BPA-induced metabolic perturbations (Fig 2C). Non-targeted lipidomics analysis provided semi-quantitative measurements of cholesterol esters (CE) and triglyceride in liver tissues. The levels of CE 20:6 and CE 19:0 were significantly elevated in the BPA group (Fig 2D). Additionally, multiple triglyceride, including TAG (18:3/18:4/18:3), DG (i-24:0/i-22:0/0:0), DG (2:0/0:0/PGJ2), DG (18:2n6/0:0/22:2n6), and MG (0:0/LTE4/0:0), were found to be significantly higher compared to the Ctrl group, indicating disrupted lipid metabolism in the liver (Fig 2E). However, no significant changes in phospholipid content were detected in the liver of the BPA group, compared to the control group (Fig 2F). Using metabolomics, we assessed the levels of fatty acid-related compounds in the liver. BPA-exposed mice exhibited significant increases in several fatty acids, including Acylcarnitine 5:0, Acylcarnitine 12:1, Acylcarnitine 10:0, Linoleoylglycine,

Linolenelaidic acid, Octadecatetraenoic acid, Eicosapentaenoic acid, and Myristic acid, compared to the Ctrl group (Fig 2G). The synthesis of triglycerides and cholesterol lipids is dependent on the presence of fatty acids. Similarly, the liver lipid content of the BPA group, including triglycerides and cholesterol, was significantly higher than that of the control group (Fig 2H-I).

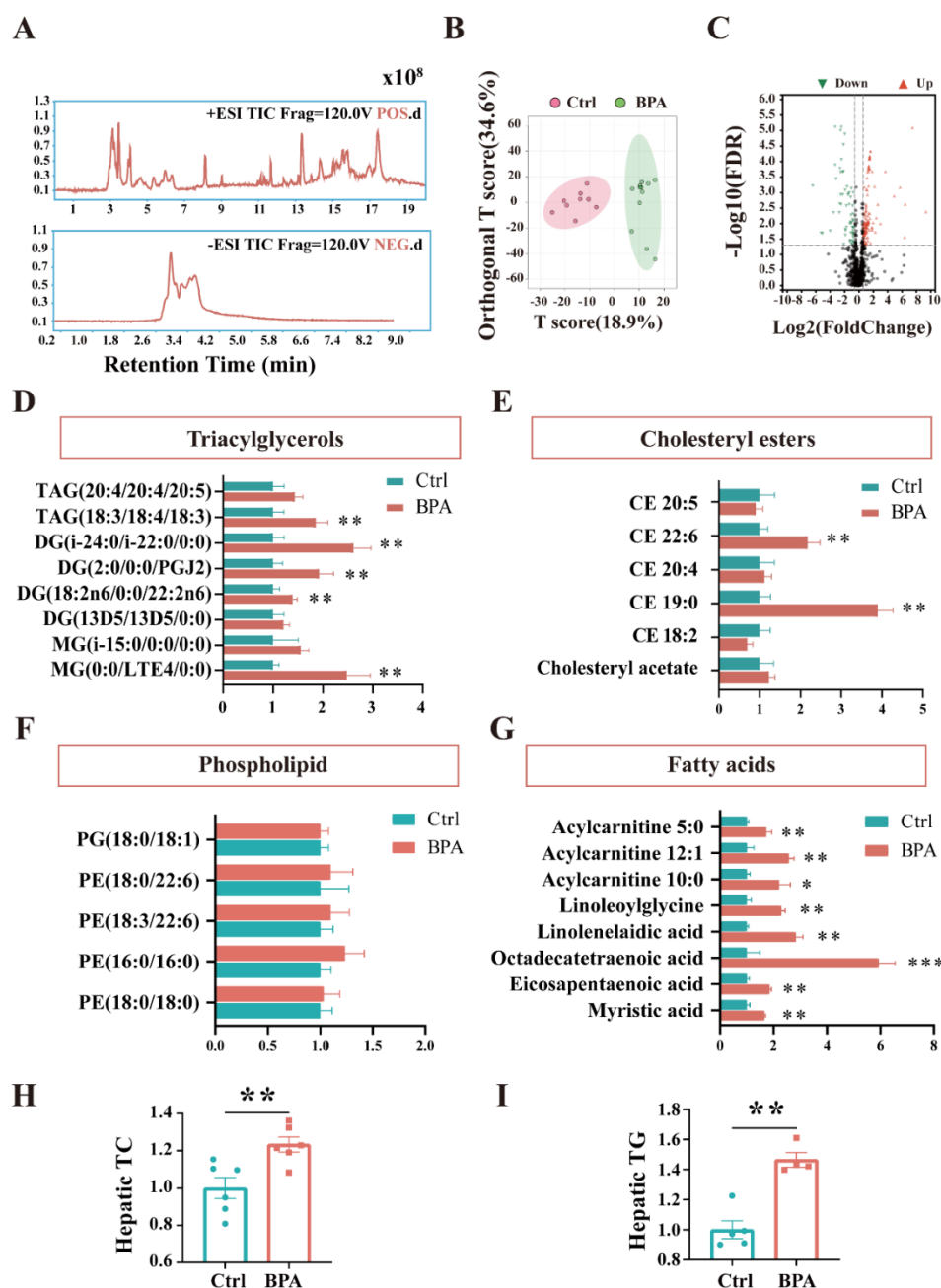


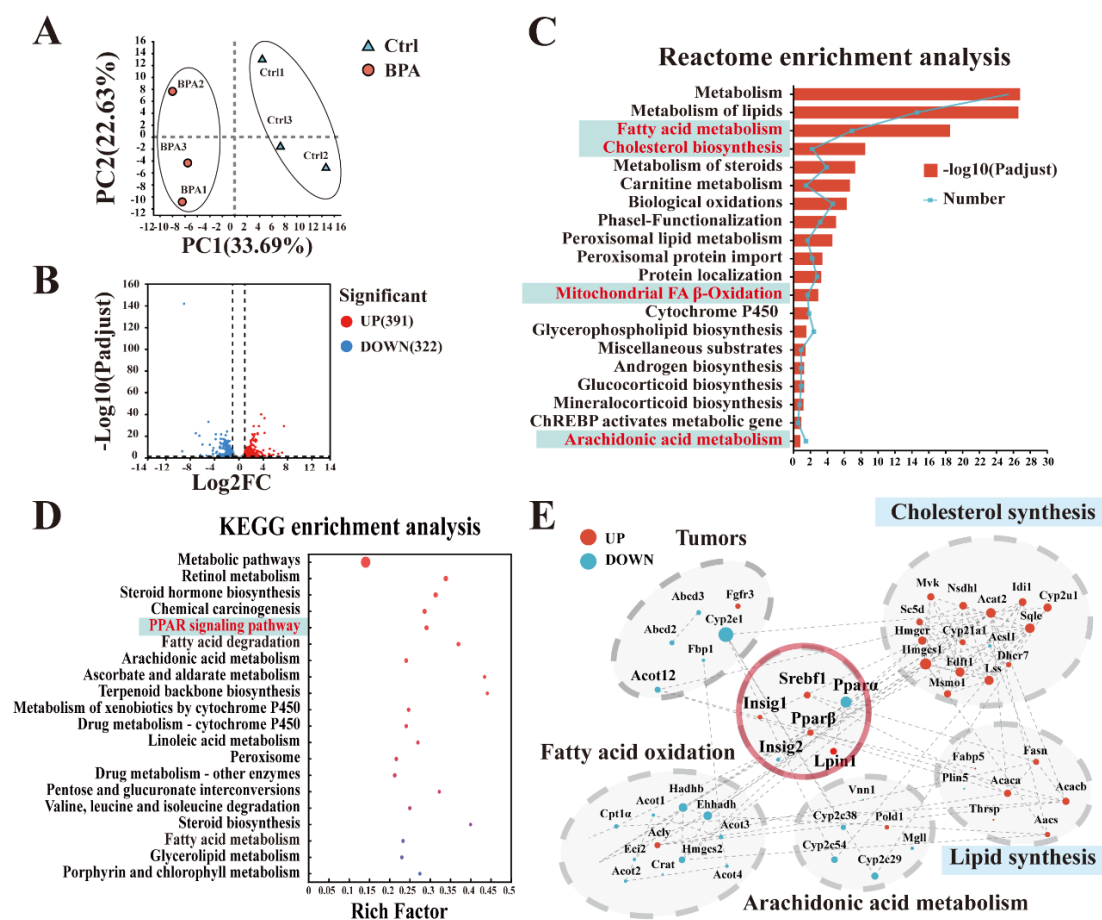
Fig.2. BPA exposure increases cholesterol and triglyceride synthesis in the liver.

Positive and negative ion chromatograms (n=8). (B) Principal component analysis (PCA) (n=8). (C) Volcano plot (n=8). (D E F) Lipidomics analysis measures cholesterol, triglyceride and phospholipid levels in the liver of mice (n=8). (G) Metabolomics measures fatty acid levels (n=8). (H I) Hepatic TC levels, hepatic TG levels (n=5 per group). The data processing method, as referenced in the figure 1 legend.

3.3 PPAR β mediates BPA-induced hepatic steatosis through transcriptional regulation of lipid metabolic pathways

The findings indicate that BPA exposure disrupts lipid homeostasis and induces hepatic steatosis by enhancing the synthesis of cholesterol esters and triglycerides. However, the precise mechanism remains

unknown. To address this, we employed transcriptomic sequencing to investigate the global molecular impacts of BPA on hepatic gene expression. The PCA plot demonstrated a clear separation between the Ctrl and BPA groups, indicating significant alterations in gene expression and metabolic pathways (Fig 3A). Additionally, the volcano plot revealed that 391 genes were significantly upregulated, while 322 genes were downregulated in the BPA group (Fig 3B). Reactome enrichment analysis revealed the 20 most significantly altered pathways, including Fatty acid metabolism, Cholesterol biosynthesis, Mitochondrial fatty acid Beta-oxidation, and Arachidonic acid metabolism (Fig 3C). Furthermore, KEGG identified the top 20 significantly altered pathways, including PPARs signaling pathway, Fatty acid degradation, Arachidonic acid metabolism, Fatty acid metabolism (Fig 3D). Notably, the Protein–protein interaction network (PPIN) indicates that the PPARs pathway is associated with cholesterol synthesis, lipogenesis, arachidonic acid metabolism, fatty acid oxidation, and tumorigenesis (Fig 3E). Investigations demonstrate that the PPARs pathway plays a critical role in regulating the transcription of key enzymes associated with lipid synthesis and metabolism^[39, 40]. This suggests that the PPARs pathway could play a central role in the development of BPA-induced hepatic steatosis in mice. We assessed the protein expression levels of three key transcription factors in the PPARs pathway in the liver. PPAR β expression was significantly reduced in the BPA group, while PPAR α and PPAR γ levels showed no significant changes (Fig 3F–G). Immunofluorescence staining also confirmed the marked decrease in PPAR β expression in the livers of BPA-exposed mice (Fig 3H–I). Based on these findings, we hypothesised that PPAR β could be a critical target molecule for BPA-induced hepatic lipotrophy in mice.



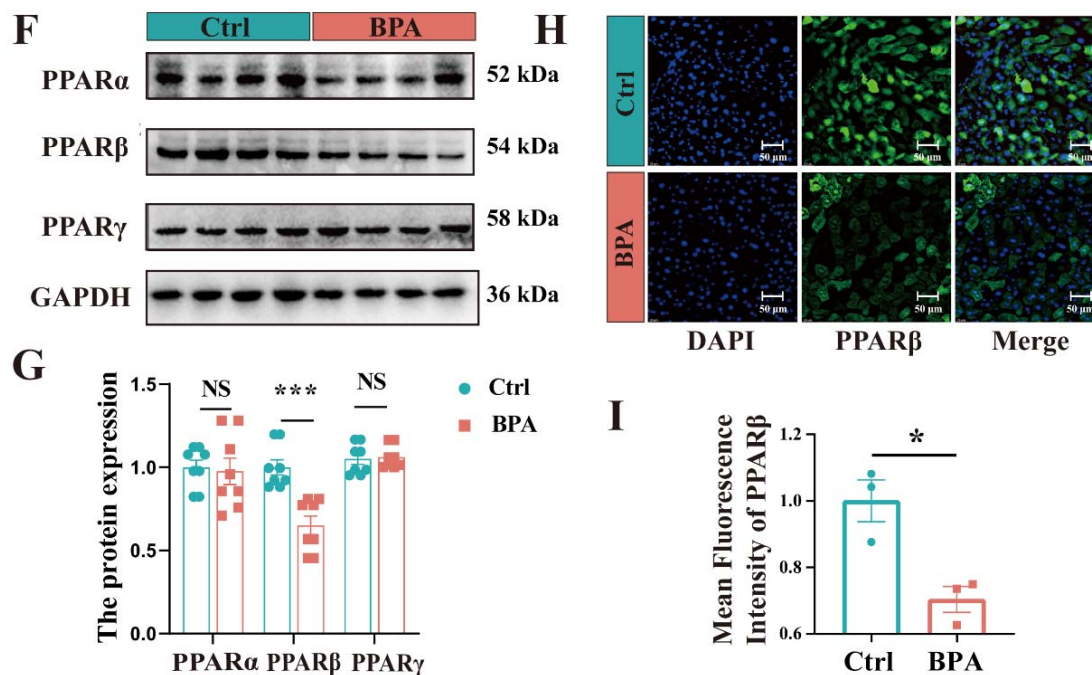


Fig.3. PPAR β involvement in regulating BPA-induced hepatic cholesterol and triglyceride metabolic disruption. Transcriptome sequencing to assess the impact of BPA exposure on gene expression in mouse liver. (A) Principal component analysis (PCA) (n=3 per group). (B) Volcano plot (n=3 per group). (C) Reactome enrichment analysis. (n=3 per group). (D) Kyoto encyclopedia of genes and genomes (KEGG) (n=3 per group). (E) The Protein–protein interaction network (PPIN) (n=3 per group). (F G) Western blot analysis and ImageJ quantification of PPARs signals (n=4 per group). (H I) Immunofluorescence Staining of PPAR β Protein Expression in Mouse Liver, with DAPI staining for cell nuclei (n=3 per group). The data processing method, as referenced in the figure 1 legend.

3.4 Hepatic PPAR β overexpression attenuates dyslipidemia and hepatic steatosis induced by BPA exposure

To establish the causal role of PPAR β suppression in BPA-mediated hepatotoxicity, we developed a liver-specific PPAR β overexpression model using AAV2/8-mediated gene delivery (Fig 4A). The results showed that PPAR β protein levels were significantly elevated in the PPAR β -OE group compared to the Ctrl group (AAV-GFP) (Fig 4B-C), indicating successful overexpression of PPAR β in the liver. Overexpression of PPAR β led to a significant reduction in serum TG and TC levels compared to the BPA group, while increasing HDL levels (Fig 4D-F). However, LDL levels showed no significant changes (Fig 4G). These results suggest that PPAR β overexpression improves lipid profile alterations caused by BPA exposure. We observed that PPAR β overexpression significantly attenuated the BPA-induced hepatic lipid accumulation, as evidenced by reduced lipid droplet intensities in oil red and H&E staining (Fig 4H-I S4A). Furthermore, physiological measurements indicated that the liver organ coefficient was significantly lower in the PPAR β -overexpressing group compared to the BPA-treated group (Fig S4B). These findings suggest that PPAR β overexpression effectively mitigates BPA-induced liver steatosis and reduces liver damage. The hepatic TG and TC quantifications revealed that the BPA+PPAR β -OE group had significantly lower levels of both TG and TC compared to the BPA group (Fig 4J-K). Additionally, overexpression of PPAR β resulted in a significant reduction in serum AST and ALT levels, indicating a mitigation of liver damage caused by BPA exposure (Fig S4C-D). These results suggest that PPAR β plays a protective role in preventing BPA-induced hepatic lipid

dysregulation and liver injury. In conclusion, the study demonstrated that hepatic PPAR β is a critical factor that regulated BPA-induced hepatic steatosis and liver damage in mice.

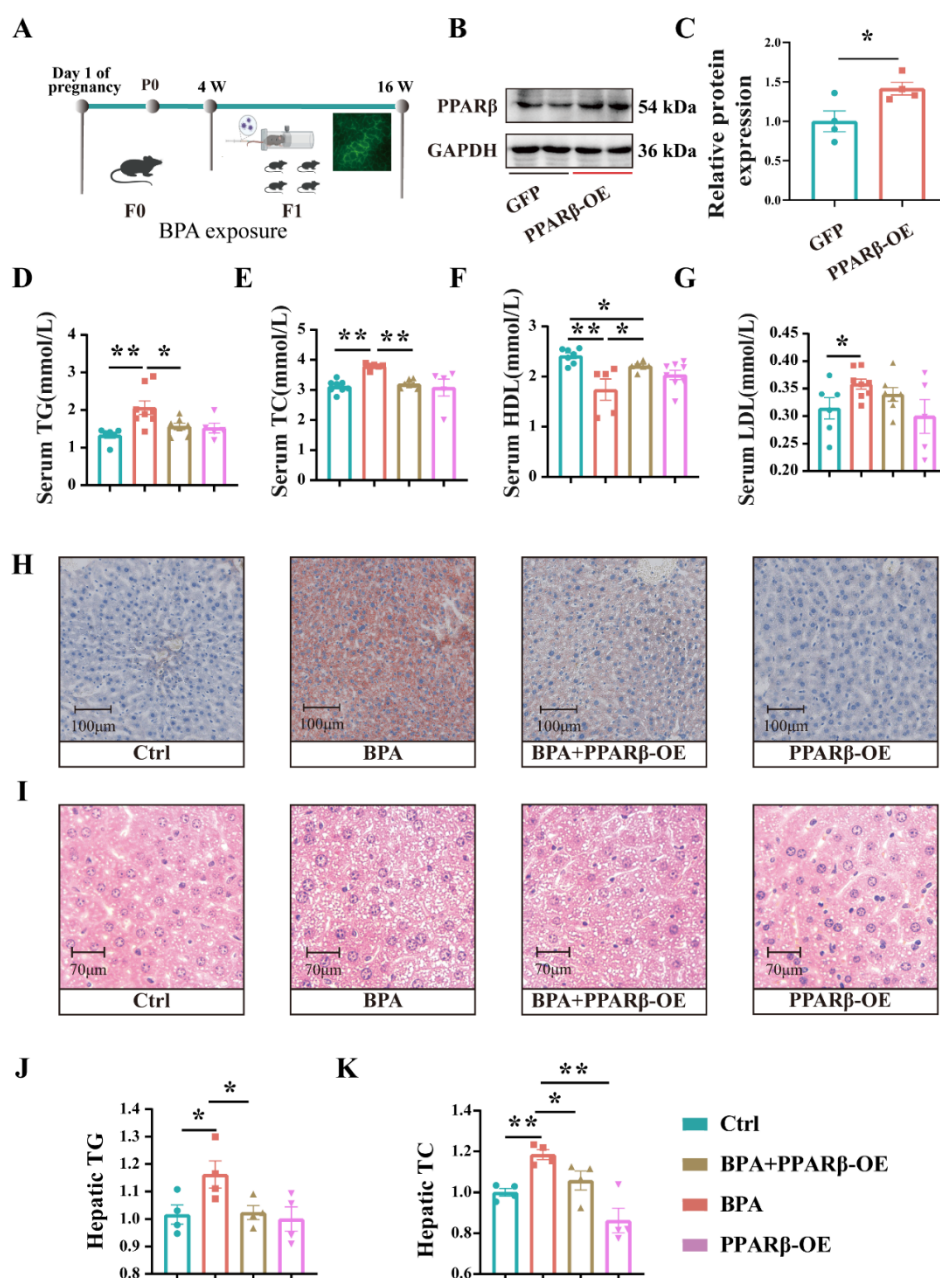


Fig. 4. PPAR β overexpression alleviates BPA-induced dyslipidemia and hepatic steatosis.

PPAR β was specifically overexpressed in the liver of mice via tail vein injection of AAV adenovirus to investigate whether PPAR β -OE can alleviate BPA-induced liver injury. (A) Paradigm of animal model treatment. (B C) Western blot analysis and ImageJ quantification of PPAR β (n=4). (D E F G) Serum lipid levels (n=5). (H) Representative micrograph of liver Oil red O stain (n=4). (I) Representative micrographs of liver H&E staining (n=4). (J K) Hepatic TG levels, hepatic TC levels (n=4).

The data processing method, as referenced in the figure 1 legend.

3.5 PPAR β Attenuates BPA-Induced Hepatic Steatosis by Modulating the INSIG/SREBP Pathway

The study further investigates the downstream mechanisms through which PPAR β -mediated BPA exposure leads to increased synthesis of triglycerides and cholesterol in mouse livers. Through bioinformatics analysis using the STRING database, we predicted the potential molecular mechanisms underlying PPAR β regulation of triglyceride and cholesterol ester synthesis. The present findings indicate that the INSIG/SREBP pathway may have a pivotal function in this process (Fig 5A-B). Recent studies have demonstrated that

PPARs promotes the transcription of INSIG [39]. Furthermore, INSIG protein interacts with SCAP to suppress the proteolytic activation of SREBP, thereby inhibiting its activation and subsequent regulation of downstream enzymes related to cholesterol and triglyceride synthesis [38]. The enzymes HMGCR and SQLE, which are involved in cholesterol synthesis, and the proteins SCD1 and FASN, which are involved in triglyceride synthesis, were significantly elevated in the BPA group (Fig 5D-E). These enzymes are subject to regulation by the transcription factors SREBP1/2, whose expression was also found to be increased (Fig 5C 5F). Furthermore, BPA has been demonstrated to reduce INSIG1/2 expression, which typically functions as an inhibitor of SREBP activation. This observation suggests an upregulation of transcriptional activity, which has been shown to disrupt lipid metabolism (Fig 5G- 5H). Consequently, the present study hypothesises that BPA exerts a suppressive effect on the expression of INSIG1/2, thereby diminishing their capacity to regulate SREBP1/ 2 activation.

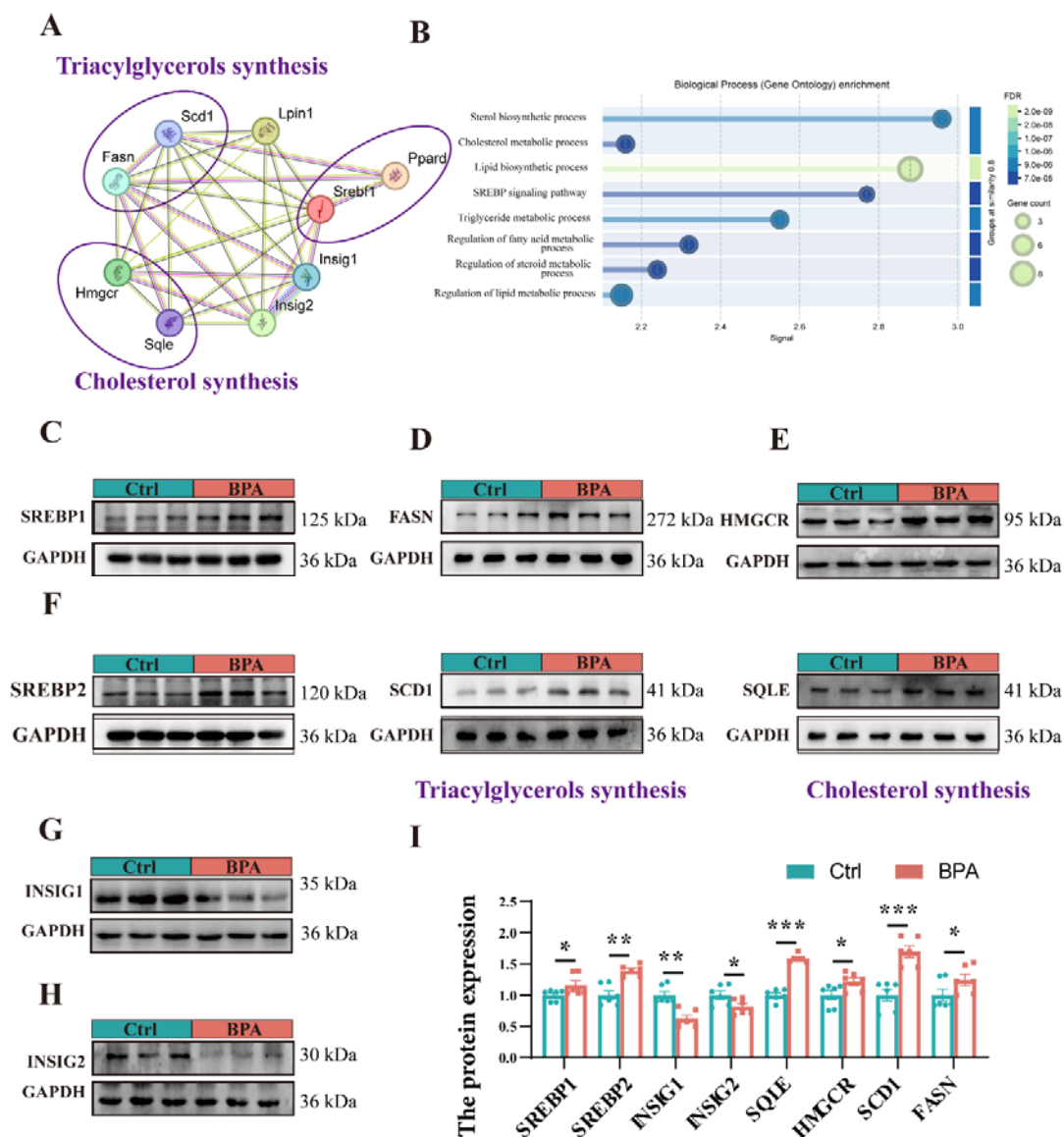


Fig.5. BPA exposure affects triglyceride synthesis and cholesterol ester synthase expression

(A B) Bioinformatics analysis using the STRING database. (C F G H) Protein expression of INSIG/SREBP pathway. (D) Protein expression of triglyceride synthesis-related molecules. (E) Protein expression of cholesterol synthesis-related molecules. (I) Densitometric quantification of WB bands. (n=3 per group). The data processing method, as referenced in the figure 1 legend.

The study further explored whether PPAR β is involved in regulating the INSIG/SREBP pathway disturbance caused by BPA exposure. Through overexpression of PPAR β in the livers of mice, we observed a significant decrease in the protein expression levels of SREBP1/2, accompanied by an increase in INSIG1/2 protein expression (Fig 6A-B). Meanwhile, PPAR β -OE group exhibited a significant decrease in the expression levels of cholesterol biosynthesis-related enzymes SQLE and HMGCR compared to the BPA group, likely contributing to lower cholesterol synthesis (Fig 6C-D). Additionally, PPAR β -OE reduced the protein expression levels of the triglyceride biosynthesis enzymes SCD1 and FASN, contributing to decreased triglyceride synthesis (Fig 6E-F).

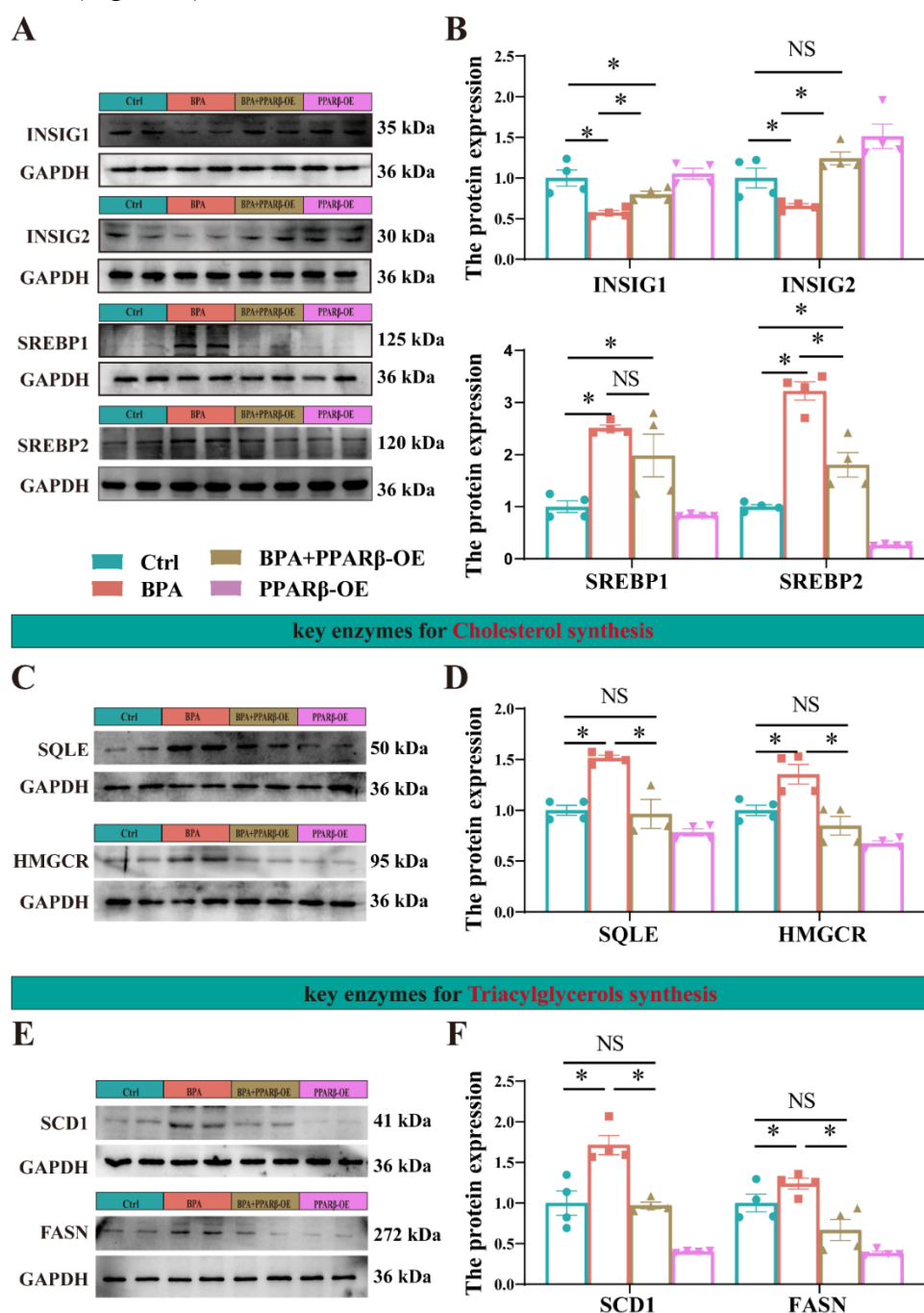


Fig.6. PPAR β -mediated regulation of the INSIG/SREBP pathway in BPA-exposed mouse liver (A B) Protein expression of INSIG/SREBP pathway and densitometric quantification of WB bands. (C D) Protein expression of cholesterol synthesis-related molecules and densitometric quantification of WB bands. (E F) Protein expression of triglyceride synthesis-related molecules and densitometric quantification of WB bands. (n=3 per group). The data processing method, as referenced in the figure 1 legend.

3.6 Hepatocyte-Specific PPAR β Suppression Mediates BPA-Induced Lipid Accumulation

Given the complexity of hepatic multicellular structures and intercellular interactions, we investigated the cell-type-specific effects of BPA by isolating three major hepatic cell populations from control and BPA-exposed (500 $\mu\text{g/kg/day}$, 8 weeks) C57BL/6 mice: hepatocytes, Kupffer cells, and hepatic stellate cells (HSCs). Notably, immunofluorescence staining revealed that PPAR β expression was significantly reduced only in hepatocytes from BPA-treated mice (Fig. 7A-B), whereas Kupffer cells and HSCs showed no detectable changes (Fig. 7C-F). This cell-type-specific PPAR β suppression was paralleled by lipid metabolic alterations: BODIPY staining demonstrated marked lipid droplet accumulation exclusively in hepatocytes (Fig. 7A-B). Kupffer cells exhibited mild lipid uptake, while HSCs remained unaffected (Fig. 7C-F). These findings demonstrate that among major liver cell types, hepatocytes are the primary targets for BPA-induced PPAR β downregulation and subsequent lipid dysregulation, independent of non-parenchymal cell interactions.

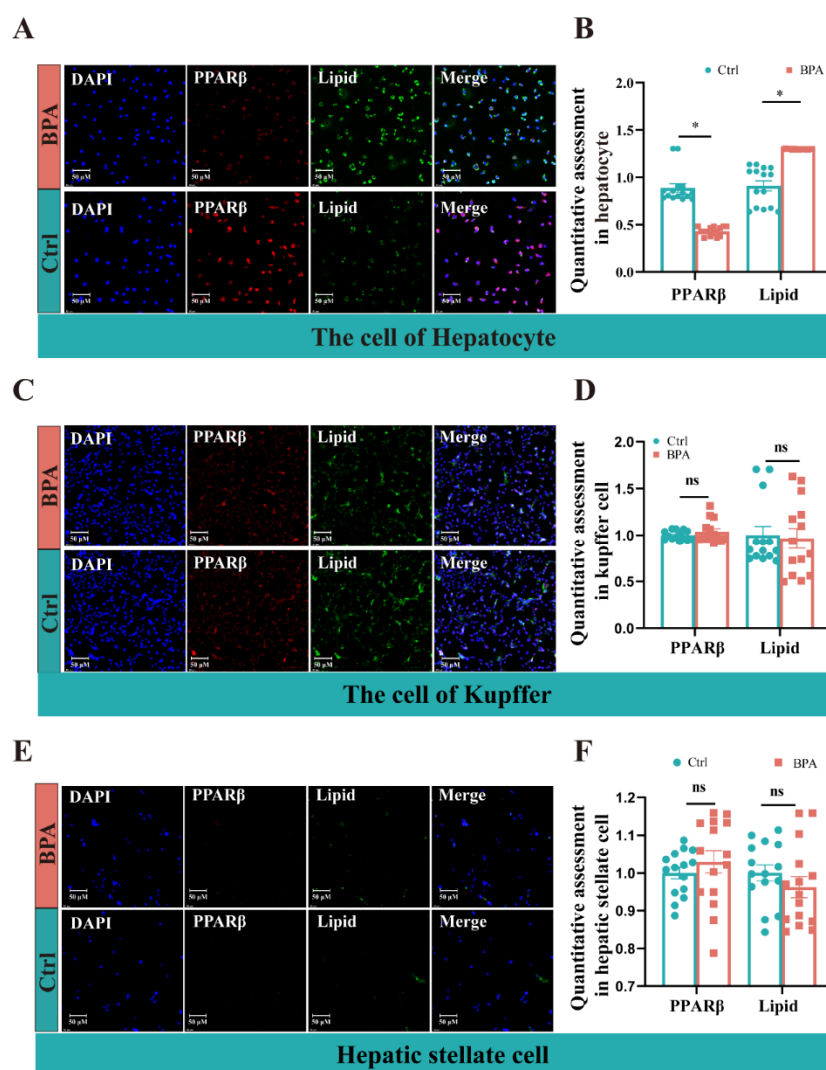


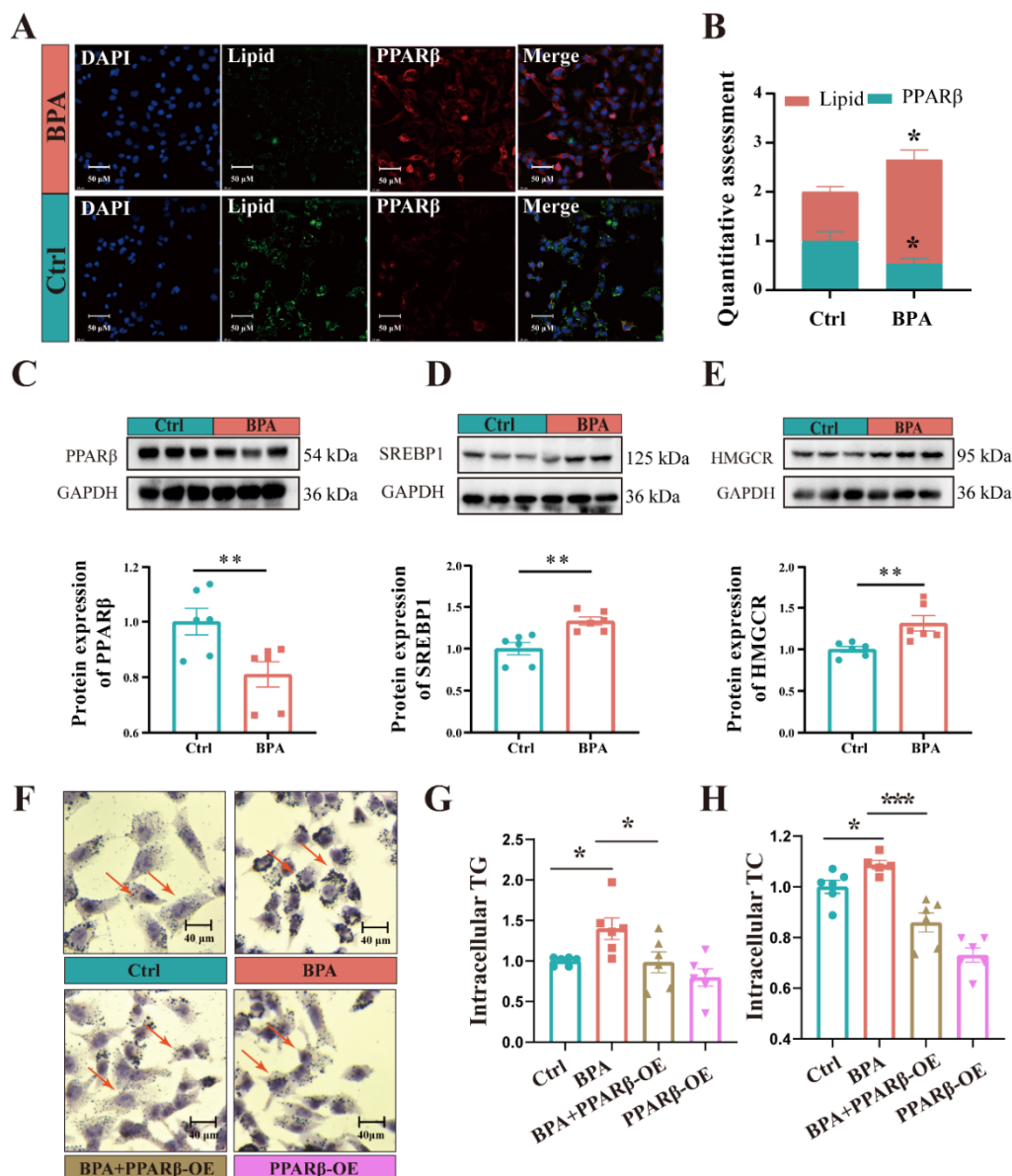
Fig. 7. BPA downregulates PPAR β and induces lipid accumulation in hepatocytes.

(A) Representative immunofluorescence images of PPAR β (red) in hepatocytes, lipid (green) from control and BPA-exposed mice. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm . (B) Quantification of PPAR β and lipid fluorescence intensity in hepatocytes. (C) Representative immunofluorescence images of PPAR β (red) and lipid (green) in Kupffer cells.

(D) Quantification of PPAR β and lipid fluorescence intensity in Kupffer cells. (E) Representative immunofluorescence images of PPAR β (red) and lipid (green) in HSCs. (F) Quantification of PPAR β and lipid fluorescence intensity in HSCs. (n=5 per group). The data processing method, as referenced in the figure 1 legend.

3.7 Validation of BPA-mediated regulation of cholesterol and glycerolipid metabolism via PPAR β in hepatocyte

In this study, Hepa1-6 cells were exposed to 10 μ M BPA for a 48-hour period, with the objective of investigating the molecular mechanisms that underpin BPA-induced lipid accumulation in hepatocytes. Immunofluorescence co-staining revealed that BPA exposure significantly reduced PPAR β protein expression, while concurrently increasing intracellular lipid accumulation (Fig 8A-B). Western blot analysis demonstrated that BPA treatment not only downregulated PPAR β but also upregulated key lipogenic proteins SREBP1 and HMGCR, mirroring our *in vivo* findings (Fig 8C-D). To functionally validate the protective role of PPAR β , we employed genetic overexpression approaches showing that PPAR β overexpression effectively attenuated BPA-induced lipid deposition, as evidenced by reduced Oil Red O staining and normalized triglyceride (TG) and total cholesterol (TC) levels (Fig 8F-H). Conversely, PPAR β knockdown in untreated cells phenocopied BPA exposure, resulting in increased lipid droplet formation and elevated TG/TC content (Fig 8I-K). These complementary experiments demonstrate that PPAR β serves as both necessary and sufficient regulator of BPA-mediated hepatic steatosis.



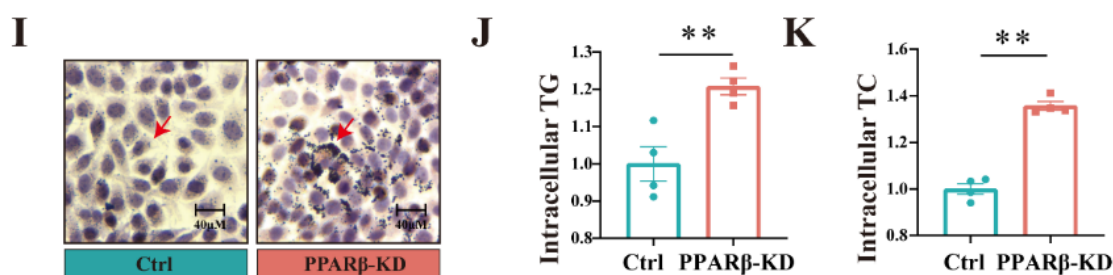


Fig.8. PPAR β overexpression alleviates BPA-induced lipid accumulation in hepatocyte

(A) Representative immunofluorescence images of PPAR β (red) in Hepa1-6 cells, lipid (green) from control and BPA-exposed mice. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μ m. (B) Quantification of PPAR β and lipid fluorescence intensity in Hepa1-6 cells (n=4). (C D E) Western blot analysis and ImageJ quantification of lipid synthesis (n=3). (F) Representative micrographs of Oil Red O staining in Hepa1-6 cells (n=3). (G H) Hepatic cholesterol levels, hepatic triglyceride levels (n=6). (I) Representative micrographs of Oil Red O staining in AMF12 cells (n=4). (J K) Hepatic cholesterol levels, hepatic triglyceride levels (n=4). The data processing method, as referenced in the figure 1 legend.

4. Discussion

This study delineates a hepatocyte-autonomous pathway by which BPA drives MASLD, addressing three fundamental gaps in environmental hepatotoxicity: Cellular selectivity: BPA specifically suppresses PPAR β in hepatocytes-but not in Kupffer cells or hepatic stellate cells-explaining parenchymal-restricted lipid accumulation; Lipid Specificity: BPA exposure induces the accumulation of specific lipid species, particularly cholesteryl esters and sphingolipids, which are linked to disrupted membrane integrity and lipotoxic injury; Transcriptional hierarchy: PPAR β regulates INSIG transcription (as established by^[41]), and BPA disrupts this axis to activate SREBP-mediated lipogenesis in our system; Collectively, these mechanisms define an endocrine disruptor-specific pathogenesis distinct from classical MASLD, positioning hepatocyte PPAR β as a sensor of chemical toxicity.

Our discovery that BPA selectively suppresses PPAR β in hepatocytes, while sparing Kupffer cells and hepatic stellate cells, challenges the prevailing view of endocrine disruptors as pan-cellular toxins^[42, 43]. The underlying mechanisms by which BPA induces a decrease in PPAR β in hepatocytes, yet spares other cell types, are not yet fully elucidated and require further investigation. The following mechanisms are posited as potential candidates. Hepatocytes exhibit higher basal PPAR β expression than KCs and HSCs (Fig. 7), creating a PPAR β -enriched molecular milieu vulnerable to BPA targeting. By contrast, non-parenchymal cells maintain lipid homeostasis through compensatory pathways: KCs utilize PPAR γ -mediated fatty acid oxidation^[44], while HSCs employ autophagy-driven lipid clearance^[45, 46], both lacking functional redundancy for the PPAR β -INSIG axis. This differential molecular vulnerability manifests as cell-type-specific metabolic outcomes. Critically, Immunofluorescence assay confirmed hepatocyte steatosis develops independently of KC-derived cytokines (Fig. 7), contrasting sharply with alcohol-induced models requiring macrophage crosstalk^[47]. BPA selective targeting of PPAR β -high hepatocytes redefines 'hepatotoxicity' as a parenchymal metabolic sabotage rather than stromal inflammatory disorder, a paradigm shift revealing environmental toxicants precision disruption of metabolic nodes.

Our finding that BPA selectively suppresses PPAR β in hepatocytes, but not PPAR α or PPAR γ , reveals a precise mechanism of endocrine disruption that differs from its reported suppression of hepatic PPAR α or

activation of adipose PPAR γ ^[48, 49]. This isoform and cell-type selectivity may be explained by several interrelated factors: the limited homology in ligand-binding domains among PPAR isoforms, which could allow BPA or its metabolites to preferentially interact with or disrupt PPAR β ^[50-52]; distinct epigenetic landscapes governing *Ppara*, *Ppard*, and *Pparg* expression in hepatocytes versus non-parenchymal cells, rendering *Ppard* uniquely susceptible to BPA-mediated epigenetic interference^[53-55]; and the utilization of compensatory pathways (e.g., PPAR γ -driven oxidation or autophagy) by Kupffer cells and hepatic stellate cells^[44], which buffers them from PPAR β modulation, thereby highlighting the hepatocyte's specific reliance on the PPAR β –INSIG axis^[56]. Thus, BPA-induced hepatotoxicity appears to stem not from a broad “pan-PPAR” disruption, but from a targeted impairment of the PPAR β node within the hepatocyte's metabolic network. This selectivity underscores the complexity of endocrine disruption and identifies PPAR β as a critical, yet previously underappreciated, hepatic sensor of chemical-induced metabolic stress.

Our data establish that BPA exposure initiates a well-defined pathogenic cascade in hepatocytes: PPAR β suppression leads to INSIG downregulation, triggering SREBP activation and subsequent upregulation of lipogenic enzymes (HMGCR, FASN). Crucially, this sequence functionally aligns with the established PPAR β –INSIG regulatory hierarchy demonstrated by ^[41], where PPAR β directly transactivates INSIG genes via promoter binding. While our study provides functional validation through gain/loss-of-function experiments, PPAR β overexpression restored INSIG expression and suppressed SREBP targets, while knockdown reciprocally disrupted this axis, the hepatocyte-specific manifestation of this cascade mirrors tissue-divergent PPAR β functions reported elsewhere ^[57-59]. The therapeutic reversibility observed (PPAR β OE normalizes lipid profiles) further confirms this axis as BPA's primary metabolic disruption point, with concordant clinical evidence showing PPAR β agonists improve dyslipidemia^[60, 61]. Thus, while direct transcriptional evidence in hepatocytes awaits further study, the mechanistic conservation across models positions the PPAR β –INSIG/SREBP pathway as a validated target for environmental steatosis intervention.

Our intervention studies demonstrate that hepatocyte-targeted PPAR β restoration completely reverses BPA-induced steatosis by rectifying the INSIG/SREBP axis, achieving a level of therapeutic efficacy unattainable with conventional approaches. Genetic overexpression (PPAR β –OE) normalized serum lipids (TG, LDL), restored INSIG1/2 expression, and suppressed lipogenic enzymes (HMGCR, FASN), while outperforming pan-PPAR agonists-rosiglitazone exacerbated lipid accumulation, and bezafibrate showed only partial efficacy versus PPAR β –OE^[62]. Critically, this targeted strategy maintained significant efficacy under physiologically relevant challenges: 1) When co-exposed to additional environmental toxicants such as phthalates, PPAR β overexpression substantially attenuated hepatic lipid accumulation^[63]; 2) In models combining metabolic comorbidities, it demonstrated superior therapeutic outcomes compared to conventional metabolic interventions. Moreover, several studies have shown that PPAR β directly regulates FAO processes^[61]. Pharmacological activation of PPAR β / δ protects mitochondrial respiratory function and enhances FAO through the PGC-1 α /NRF-1 pathway^[64, 65]. In addition, PPAR β has been shown to be involved in the AMPK-mediated FAO process in mouse liver^[58]. Similarly, it was found that BPA exposure led to

altered expression of genes related to FAO, such as *Ehhadh*, *Cpt2*, *Acaa1b*, *SCD1*, *Srebfl*, and *Acly*, among others, while PPAR β knockdown exacerbated the disruption of FAO-related gene expression in hepatocytes caused by BPA exposure in vitro experiment (Fig S5). These findings establish a stratified intervention paradigm: patients with PPAR β suppression benefit from receptor-specific agonism, while others respond to lifestyle modification^[66-68]. The complete metabolic rescue achieved through singular transcriptional node modulation reveals a foundational principle: environmental metabolic disruption retains epigenetic plasticity exploitable for ecological precision medicine, where exposome signatures (e.g., CE 20:6) guide targeted therapy against chemical hepatotoxicity^[69].

This study demonstrates that chronic BPA exposure induces MASLD through hepatocyte-specific suppression of PPAR β , which disrupts the INSIG/SREBP regulatory axis and drives pathogenic lipid accumulation. It has been established that BPA selectively downregulates PPAR β in hepatocytes, but not in Kupffer cells or hepatic stellate cells. This results in a cascade where diminished INSIG expression activates SREBP transcription factors, ultimately upregulating cholesterol (HMGCR, SQLE) and triglyceride (SCD1, FASN) synthesis enzymes. Lipidomic profiling identified cholesteryl ester CE 20:6 and triglyceride TAG 18:3/18:4/18:3 as signature lipid species elevated. Crucially, hepatic PPAR β overexpression reversed the toxicity of BPA by restoring the suppression of SREBP by INSIG, normalising serum lipids and reducing hepatic lipid deposition. In contrast, knockdown of the same receptor replicated the steatotic effects of BPA, thus confirming the necessity of the latter in maintaining lipid homeostasis. These findings position PPAR β as both a molecular sensor for BPA exposure and a therapeutic target for chemical-induced metabolic disease.

5. Conclusion

In conclusion, this study establishes that chronic BPA exposure drives metabolic-associated fatty liver disease through the hepatocyte-specific suppression of PPAR β (Fig. 9). This key event disrupts the PPAR β -INSIG-SREBP regulatory axis, leading to unchecked lipogenesis and the pathological accumulation of specific lipids. Genetic restoration of PPAR β completely reverses the steatotic phenotype, confirming its central role. Our work defines an endocrine-disruptor-specific form of MASLD and identifies hepatocyte PPAR β as both a critical biomarker for chemical toxicity and a validated therapeutic target, providing a mechanistic foundation for addressing environment-related metabolic liver disease.

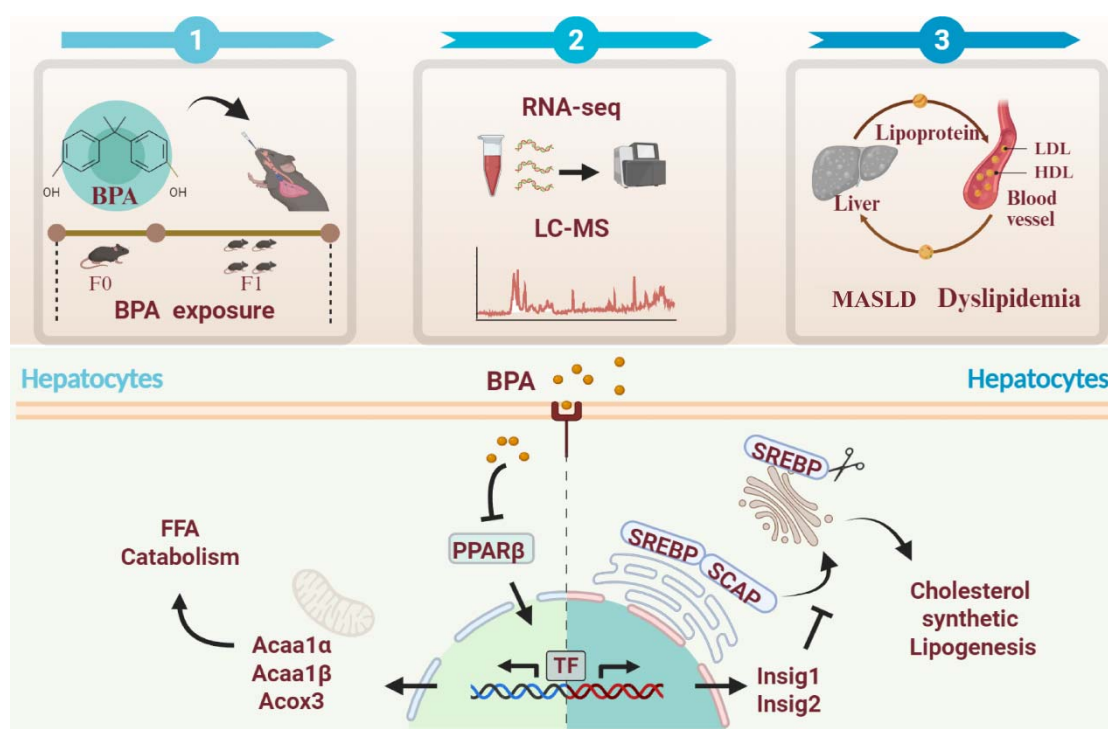


Fig. 9 Created with BioRender.com The licensing agreement number: VD298ZQKD6.

BPA exposure promotes MASLD in mice. The proposed mechanism involves BPA-mediated suppression of PPAR β expression in hepatocytes. This downregulation alters transcriptional programs, leading to increased synthesis of triglycerides and cholesteryl esters, coupled with decreased fatty acid oxidation, thereby driving hepatic steatosis.

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.

Funding Declaration

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