

Chronic Treatment With WY-14643 Induces Tumorigenesis and Triglyceride Accumulation in Mouse Livers[§]

Jie Yang,¹ Shicheng Fan,¹ Yifei Zhang, Min Huang, Yue Gao, and Huichang Bi

Guangdong Provincial Key Laboratory of New Drug Design and Evaluation, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, China (J.Y., S.F., Y.Z., M.H., Y.G., H.B.); and NMPA Key Laboratory for Research and Evaluation of Drug Metabolism, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, Guangdong, China (S.F., H.B.)

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ABSTRACT

Peroxisome proliferator-activated receptor α (PPAR α) is closely related to lipid metabolism and various liver diseases. Previous study has shown that chronic treatment with PPAR α agonist WY-14643 can induce liver tumors in rodents, but the implications of this process on lipid metabolism in the liver remain unclear. Thus, this study aimed to explore the influences of chronic treatment with WY-14643 on the liver and hepatic lipid metabolism. Wild-type C57BL/6 mice were treated with WY-14643 (100 mg/kg/week, i.p.) or corn oil, and liver and serum samples were collected for testing after 42 weeks of WY-14643 treatment. The results showed that hepatomegaly, liver tumors with mild liver injury, and hepatocyte proliferation were induced in mice treated with WY-14643. The mRNA and protein expression levels of PPAR α downstream targets acyl-CoA oxidase 1 and cytochrome P450 4A were significantly upregulated in the WY-14643-treated group. Lipidomic analysis revealed that chronic treatment with WY-14643 disturbed

lipid homeostasis, especially triglycerides (TGs), which were significantly elevated after WY-14643 treatment. Moreover, TG homeostasis-related genes were significantly increased in the WY-14643-treated group. In conclusion, these findings demonstrated that hepatomegaly and liver tumors induced by chronic treatment with WY-14643 in mice are accompanied by hepatocyte proliferation and TG accumulation.

SIGNIFICANCE STATEMENT

The present study clearly demonstrated that sustained peroxisome proliferator-activated receptor α (PPAR α) activation by chronic treatment with WY-14643 induces hepatomegaly and liver tumors with triglyceride accumulation by regulating lipid homeostasis-related genes in mice. These findings may help to clarify the influences of sustained PPAR α activation on liver lipid homeostasis and provide data for the clinically rational use of drugs that can activate PPAR α .

Introduction

Peroxisome proliferator-activated receptor α (PPAR α ; NR1C1), a ligand-activated nuclear receptor, is highly expressed in the liver (Isse-mann and Green, 1990; Lefebvre et al., 2006). The ligands of PPAR α are a plethora of exogenous or endogenous compounds called peroxisome proliferators (PP), ranging from herbicides, experimental (WY-

14643), or marketed pharmaceutical agents (fibrates) to fatty acids (FAs) and their metabolites, which can significantly increase the number and size of peroxisomes in the liver (Kliwer et al., 1999; Kersten, 2014). After being activated, PPAR α transfers to the nucleus and plays a variety of physiologic and pathologic regulatory roles by regulating the expression of its downstream genes involved in lipid and glucose homeostasis, cell proliferation, obesity, cancer, and inflammation (Bishop-Bailey, 2000; Chinetti et al., 2003).

Short-term treatment of PPAR α activators can induce hepatomegaly (Peters et al., 1997; Corton et al., 2000; Tien et al., 2003). Chronic treatment with PP can lead to hepatomegaly and ultimately liver tumors in rodents (Reddy et al., 1980). Studies have shown that *Ppara*-null mice treated with PP did not induce liver tumors, indicating that PP-induced hepatocarcinogenic effects are dependent on PPAR α activation (Lee et al., 1995; Peters et al., 1997). Several mechanisms underlying the carcinogenesis of PP have been proposed. First, different degrees of induction of acyl-coenzyme A oxidase 1 (ACOX1) and catalase can induce liver tumors via increasing production of reactive oxygen intermediates (Nemali et al., 1989; O'Brien et al., 2005). PP can also influence the cell cycle and apoptosis (Roberts et al., 1995). Studies have shown that mitogenic and hepatocarcinogenic effects caused by PP may be

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¹J.Y. and S.F. contributed equally to this work.

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ABBREVIATIONS: ACOX1, acyl-coenzyme A oxidase 1; AFP, α -fetoprotein; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CCND1, cyclin D1; CDK, cyclin-dependent kinase; CTNNB1, β -catenin; CV, central vein; CYP4A, cytochrome P450 4A; FA, fatty acids; FXR, farnesoid X receptor; GS, glutamine synthetase; KRT8, cytokeratin 8; NR, nuclear receptor; PC, phosphatidylcholine; PCNA, proliferation cell nuclear antigen; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PP, peroxisome proliferators; PPAR α , peroxisome proliferator-activated receptor α ; PS, phosphatidylserine; PV, portal vein; PXR, pregnane X receptor; TG, triglyceride; VIP, variable importance in projection.

attributed to the downregulation of let-7C microRNA, which then contributes to the induction of myelocytomatosis oncogene (Shah et al., 2007).

Lipids are vital for normal cellular and systemic functions (Bartlett and Eaton, 2004; Nguyen et al., 2008). Precise regulation of hepatic lipid homeostasis is orchestrated by multiple factors. PPAR α has been reported to be related to multiple aspects of lipid homeostasis (Lee et al., 1995; Kersten, 2014), including FA degradation, synthesis, and transport (Kroetz et al., 1998; Kersten et al., 1999; Patsouris et al., 2004). The phenotype of *Ppar α* -deficient mice is normal, but these mice are susceptible to many metabolic defects. Recent studies revealed

that hepatocyte-restricted PPAR α deletion blunts fasting-induced ketogenesis (R  gnier et al., 2018), impairs FA homeostasis, and promotes nonalcoholic fatty liver disease during aging (Montagner et al., 2016). Thus, PPAR α represents a major therapeutic target for lipid metabolism disorders (Gross et al., 2017).

It has been reported that lipid metabolism is altered in hepatocellular carcinoma (Pope et al., 2019). However, it is still unclear how lipid metabolism changes during sustained PPAR α activation. Thus, this study aimed to explore the influences of sustained PPAR α activation by chronic treatment with WY-14643 on the liver and hepatic lipid metabolism. This study demonstrated that hepatomegaly and liver tumors

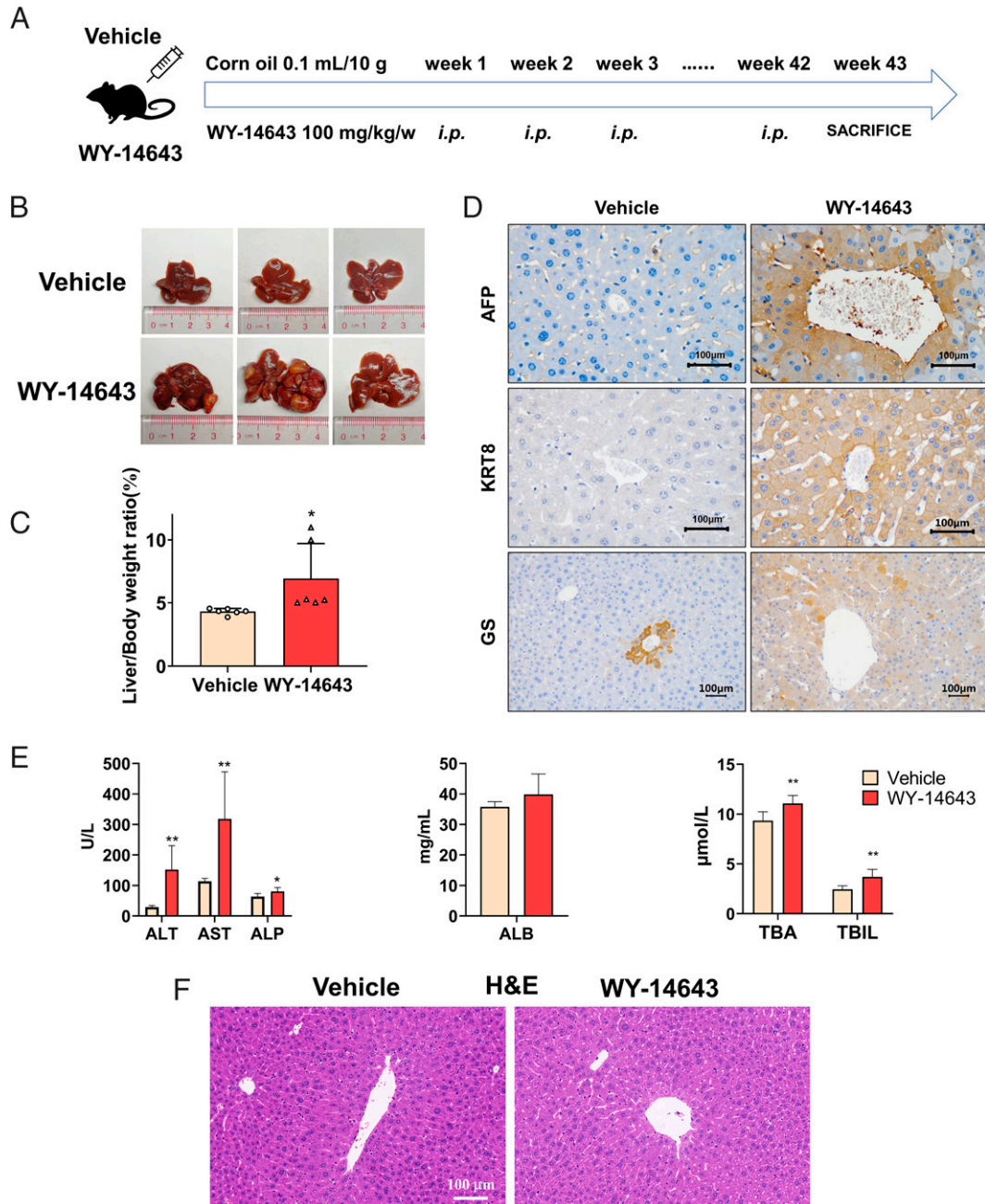


Fig. 1. Chronic treatment with WY-14643 induces hepatomegaly and hepatocarcinogenesis. (A) Scheme for the animal experiment. (B) Morphologic photographs of representative livers. (C) Liver-to-body weight ratios. (D) Immunohistochemical staining of AFP, KRT8, and GS. Scale bar = 100 μ m. (E) The concentrations of serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, total bile acid, and total bilirubin in the vehicle and WY-14643-treated groups. (F) Representative H&E-stained liver sections. Scale bar = 100 μ m. Data are presented as the means \pm S.D. (n = 6). Comparisons are based on the two-tailed Student's t test. * P < 0.05, ** P < 0.01 compared with the vehicle group.

induced by chronic treatment with WY-14643 in mice were accompanied by triglyceride (TG) accumulation, providing new insights into the effects and clinically rational use of PPAR α agonists for chronic treatment.

Materials and Methods

Chemicals and Reagents. WY-14643 (catalog no. A4305) and corn oil (catalog no. C116025) were acquired from APExBIO Technology (Houston, TX, USA) and Aladdin Company (Shanghai, China), respectively. Anti-acyl-coenzyme A oxidase 1 (anti-ACOX1; catalog no. D121471), anti-cyclin D1 (anti-CCND1; catalog no. D120509), anti-cyclin-dependent kinase 4 (anti-CDK4; catalog no. D120396), and anti-cytokeratin 8 (anti-KRT8; catalog no. D220230) antibodies were from Sangon Biotech (Shanghai, China). Anti-cytochrome P450 4 (anti-ACYP4A; catalog no. sc-271983), anti- α -fetoprotein (anti-AFP; catalog no. sc-130302), and anti-PPAR α (catalog no. sc-398394) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). The anti- β -catenin antibody (anti-

CTNNB1; catalog no. 610153) was from BD Biosciences (San Jose, CA, USA). Anti-glutamine synthetase (anti-GS; catalog no. A5437) and anti-constitutive androstane receptor (anti-CAR; catalog no. A17066) antibodies were from ABclonal Technology (Wuhan, China). Rabbit monoclonal anti- β -ACTB (catalog no. 4970), and anti-proliferation cell nuclear antigen (anti-PCNA; catalog no. 13110) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-farnesoid X receptor (anti-FXR; catalog no. Ab129089) and anti-pregnane X receptor (anti-PXR; catalog no. ab192579) antibodies were acquired from Abcam (Cambridge, UK). NCM Universal Antibody Diluent (catalog no. WB500D) for Western blot was purchased from NCM Biotech (Suzhou, China).
Animal Experiment. The study was carried out on male C57BL/6 mice. For the age, origin, and feeding conditions of animals, refer to our previously published article (Zhang et al., 2022). The animal experiment was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. These animals were randomly divided into the vehicle group or WY-14643 group (6/group) and were injected intraperitoneally with vehicle corn oil or WY-14643

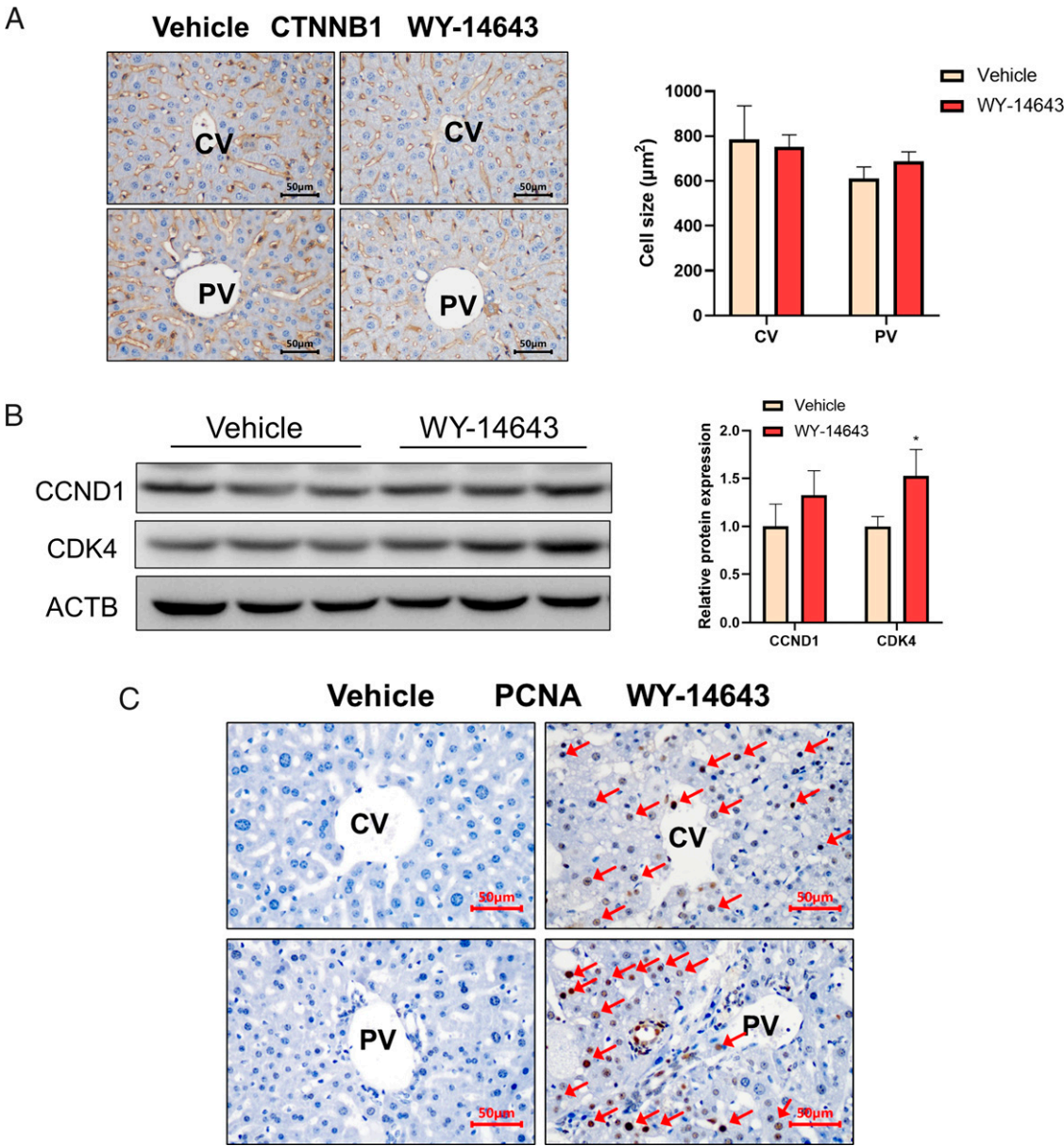


Fig. 2. Chronic treatment with WY-14643 induces hepatocyte proliferation without hepatocyte enlargement. (A) Immunohistochemical staining of CTNNB1 ($n = 3$). Scale bar = 50 μm . (B) The hepatic protein expression levels of the proliferation-related proteins CCND1 and CDK4. (C) Immunohistochemical staining of PCNA. Scale bar = 50 μm . Red arrows: brown-stained PCNA-positive cells. Data are presented as the means \pm S.D. Comparisons are based on the two-tailed Student's t test. * $P < 0.05$ compared with the vehicle group.

(100 mg/kg/week), respectively, for 42 weeks. All mice were euthanized one week after the last treatment. For the collection and storage of animal samples, refer to our previously published article (Zhao et al., 2021).

Biochemical and Histologic Analysis. The measurement of alanine aminotransferase, alkaline phosphatase, albumin, aspartate aminotransferase, total bile acid, and total bilirubin, and hepatic TG contents were performed according to our previously published methods (Jiao et al., 2020; Zhang et al., 2022). Liver tissues were stained for H&E, KI67, CTNNB1, α -fetoprotein, KRT8, and GS. The H&E staining and immunohistochemical staining were conducted according to our previously published article (Jiao et al., 2020).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction Analysis. Total RNA was extracted from liver tissue by Trizol. The kits used for obtaining cDNA (catalog no. AG11711) and quantitative polymerase chain reaction (catalog no. AG11701) were obtained from Accurate Biotechnology (Hunan, China). The primer sequences used in this study are shown in Supplementary Table 2. The fold changes of mRNA levels were analyzed by the $\Delta\Delta C_t$ method.

Total Liver Protein Extraction and Western Blot. The procedures of protein extraction and Western blot analysis were accorded to the previously published methods (Zeng et al., 2017; Jiang et al., 2019; Zhang et al., 2022).

Lipidomic Analysis. Following the previously published protocol (Jiang et al., 2021; Zhang et al., 2021), lipids in 30 mg homogenized liver samples were extracted by a 1.2 mL methanol/methyl tert-butyl ether/H₂O (4:5:5, v/v/v) mixture. After vortex and centrifugation, the organic phase was collected in new Eppendorf tubes and then evaporated until dry in a vacuum dryer. The evaporated extract was redissolved in 200 μ L methanol/isopropanol (1:1, v/v) and then centrifuged. UHPLC-Q Exactive system (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze a 2 μ L supernatant of each sample. The details of chromatographic separation, mass spectrometry analysis, and data processing were referred from the previously published article (Jiao et al., 2020).

Statistical Analysis. All data were presented as mean \pm S.D. Two-tailed Student's *t* test was used to assess the data processing by GraphPad Prism 8.2.1 software (GraphPad Software, Inc., San Diego, CA, USA) and SPSS 23.0 software (IBM Analytics, Armonk, NY, USA). Significance is represented by **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the vehicle.

Results

Chronic Treatment With WY-14643 Induces Hepatomegaly and Liver Tumors. Our previous study showed that intraperitoneal injection of WY-14643 (100 mg/kg) for 10 days significantly induced

liver enlargement in mice (Fan et al., 2022). To elucidate the effects of chronic WY-14643 treatment on the liver, mice were injected with vehicle corn oil or WY-14643 (100 mg/kg/week, i.p.) for 42 weeks (Fig. 1A). The results showed that the liver-to-body weight ratio was significantly increased by chronic treatment with WY-14643 (from $4.31\% \pm 0.24\%$ to $6.93\% \pm 2.79\%$, +60.76%), and two of six mice in the WY-14643 group developed visible liver tumors (Fig. 1, B and C). Immunohistochemical staining of three well-established tumor markers showed that the numbers of AFP-, GS-, and KRT8-positive hepatocytes were increased significantly in the WY-14643-treated group (Fig. 1D). Serum alkaline phosphatase, total bile acid, aspartate aminotransferase, alanine aminotransferase, and total bilirubin levels were slightly increased after WY-14643 treatment, while no significant differences were observed in serum albumin levels (Fig. 1E) or the H&E staining images (Fig. 1F) of the two groups. The results indicated that liver enlargement and liver tumors induced by chronic treatment with WY-14643 were accompanied by mild liver injury.

Chronic Treatment With WY-14643 Induces Hepatocyte Proliferation Without Hepatocyte Enlargement. Our previous study showed that 10 days of treatment with WY-14643 induced hepatocyte enlargement and proliferation around the central vein (CV) area and the portal vein (PV) area, respectively (Fan et al., 2022). To investigate the effects of chronic treatment with WY-14643 on hepatocyte size and proliferation, CTNNB1 and PCNA staining, as well as Western blot analysis of CCND1 and CDK4, were performed. CTNNB1 staining showed that the hepatocyte size did not change around both the CV and PV areas after chronic WY-14643 treatment (Fig. 2A). The expression levels of CDK4 and CCND1 showed a rising tendency, although the expression level of CCND1 did not alter significantly (Fig. 2B). PCNA staining revealed that hepatocyte proliferated around both the CV and PV areas after chronic treatment with WY-14643 (Fig. 2C). These results demonstrated that chronic treatment with WY-14643 induced hepatocyte proliferation without hepatocyte enlargement.

Chronic Treatment With WY-14643 Upregulates Downstream Targets of PPAR α . To assess the effects of chronic treatment with WY-14643 on PPAR α activation, the expression levels of PPAR α

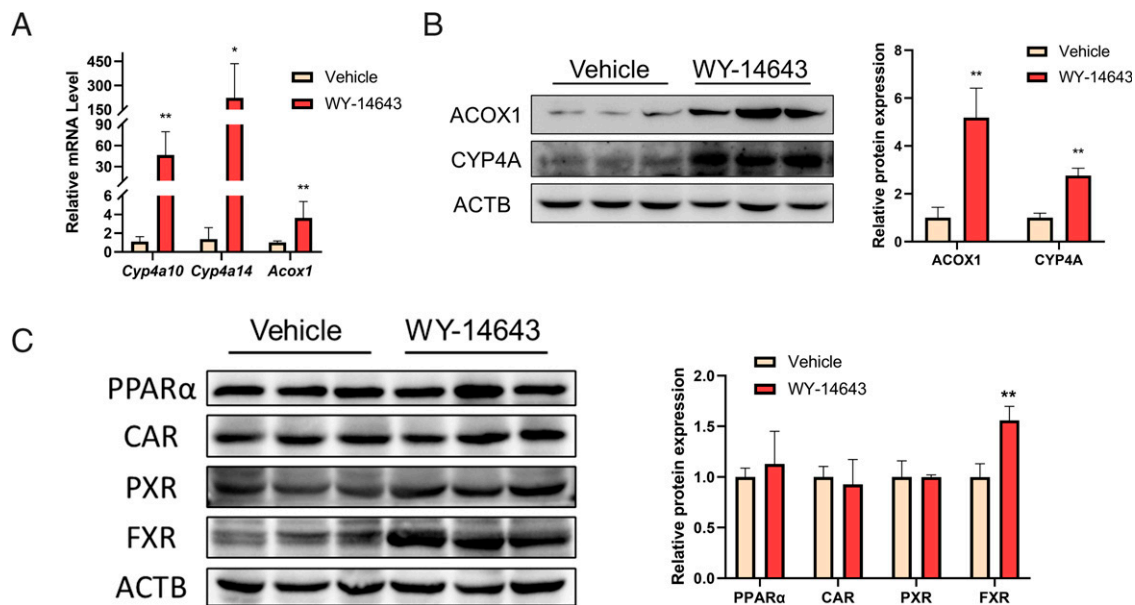


Fig. 3. Chronic treatment with WY-14643 upregulates downstream targets of PPAR α . (A) The mRNA expression levels of the PPAR α downstream targets *Cyp4a10*, *Cyp4a14*, and *Acox1* (*n* = 6). (B) The hepatic protein expression levels of the PPAR α downstream targets ACOX1 and CYP4A (*n* = 3). (C) The hepatic protein expression levels of PPAR α , CAR, PXR, and FXR (*n* = 3). Data are presented as the means \pm S.D. Comparisons are based on the two-tailed Student's *t* test. **P* < 0.05, ***P* < 0.01 compared with the vehicle group.

downstream targets were measured. The results indicated that chronic treatment with WY-14643 significantly increased the mRNA expression levels of *Cyp4a10*, *Cyp4a14*, and *Acox1* by 41.63-, 161.73-, and 2.59-fold, respectively (Fig. 3A). The protein expression levels of ACOX1 and CYP4A were increased by 4.18- and 1.76-fold, respectively (Fig. 3B). However, the protein expression level of PPAR α did not change (Fig. 3C). The results demonstrated that chronic treatment with WY-14643 upregulates downstream targets of PPAR α .

Due to the possible crosstalk between different nuclear receptors (NRs), the protein expression levels of several other NRs associated with hepatomegaly, including CAR, PXR, and FXR, were detected. The results showed that the expression of CAR and PXR was not altered (Fig. 3C). However, the expression level of FXR was increased significantly by 0.56-fold (Fig. 3C).

Chronic Treatment With WY-14643 Affects the Hepatic Lipid Profile and Induces TG Accumulation in Mouse Livers. To evaluate the effects of chronic treatment with WY-14643 on the hepatic lipid profile, lipidomic analysis was performed. The principal component analysis score plots (Fig. 4A) and orthogonal partial least-discriminant analysis score plots (Fig. 4B) both showed that the lipid profiles of the

two groups clustered well and exhibited considerable separation in positive and negative ion modes, indicating distinct lipid profiles between the two groups.

Next, LipidSearch software was used to identify the changed lipids between the two groups. Here, 923 ions were identified, and 43 potential hepatic lipid molecules were screened (Supplemental Table 1). Lipids with variable importance in projection values > 1.0 and $P < 0.05$ are shown in the volcano plot (Fig. 4C). Dots highlighted with red or green indicated lipids that had increased or decreased more than threefold, respectively, and these lipids are also shown in the heatmap (Fig. 4D). As shown in Fig. 4D, 39 lipids were increased significantly in the WY-14643 group, all of which belong to the TG class, and 4 lipids were decreased, including 2 from the phosphatidylethanolamine (PE) class, 1 from the phosphatidylinositol (PI) class and 1 from the phosphatidylserine (PS) class. These findings indicated that chronic treatment with WY-14643 significantly affected the hepatic lipid profile and induced TG accumulation in mouse livers.

Chronic Treatment With WY-14643 Induces Hepatic TG Accumulation by Regulating TG Homeostasis-Related Genes. Lipidomic analysis indicated that chronic treatment with WY-14643

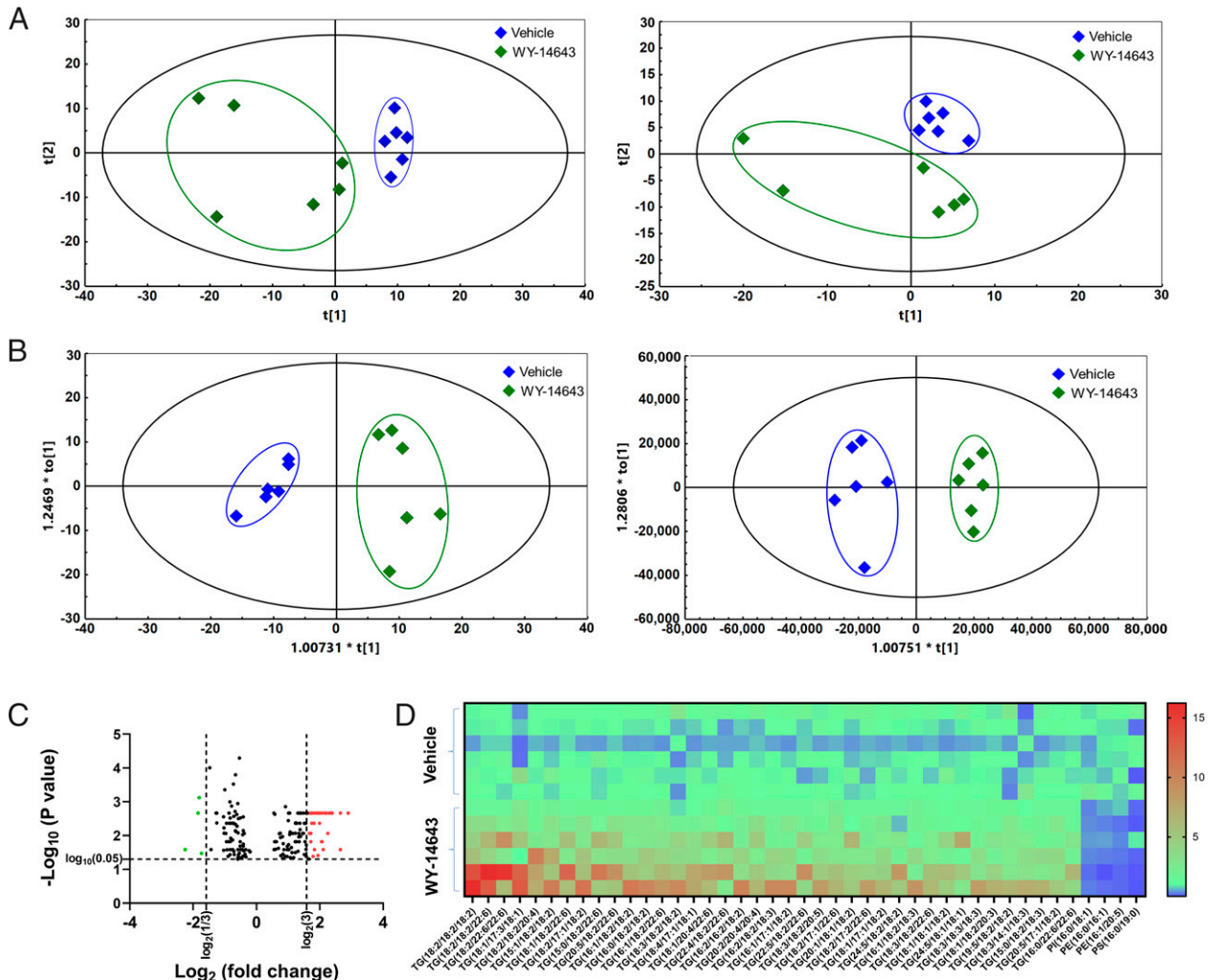


Fig. 4. Chronic treatment with WY-14643 affects the hepatic lipid profile and induces TG accumulation in mouse livers. (A) Principal component analysis score scatter plots and (B) orthogonal partial least-discriminant analysis score plots of the lipidomic profiles in positive (left) and negative (right) ion modes for the vehicle and WY-14643 groups. (C) Volcano plot of the altered lipids [red, lipids increased more than threefold with $P < 0.05$, variable importance in projection (VIP) > 1.0; green, lipids decreased more than threefold with $P < 0.05$, VIP > 1.0]. (D) Heatmap of 43 lipids selected by VIP > 1.0, $P < 0.05$ and fold change > 3.0. Comparisons are based on the two-tailed Student's t test and the Mann–Whitney U test.

induced TG accumulation in mouse livers; thus, oil red O staining was performed for confirmation. The results demonstrated a pronounced increase in lipid droplets in the hepatocytes after WY-14643 treatment (Fig. 5A). Hepatic TG content was then further tested. The results demonstrated that the hepatic TG level in the WY-14643 group was elevated by 0.67-fold versus the vehicle group (Fig. 5B). The mRNA levels of hepatic TG homeostasis-related genes were further tested (Fig. 5C). The expression levels of *Cd36* and fatty acid binding protein 1 (*Fabp1*), involved in FA uptake, were elevated 3.39- and 5.04-fold after WY-14643 treatment. Acyl-CoA synthetase long chain family member 1 (*Acs1l*), related to TG synthesis, was significantly increased by 4.55-fold after WY-14643 treatment. Furthermore, genes related to FA metabolism, such as *Acox1*, *Hsl* (hormone-sensitive lipase), and *Ppar α* , were significantly upregulated 2.65-, 5.58-, and 3.36-fold after WY-14643 treatment, respectively. These results indicated the possibility that the TG accumulation induced by chronic treatment with WY-14643 was mediated by these TG homeostasis-related genes.

Taken together, the lipidomics data and gene expression demonstrated that chronic treatment with WY-14643 exerts profound effects on mouse hepatic lipid homeostasis. Thus, the accumulation of hepatic TG induced by WY-14643 may contribute to liver enlargement.

Discussion

Normally, liver size is constant and strictly orchestrated to maintain body homeostasis. Chronic treatment with WY-14643, a PPAR α agonist, has been reported to induce liver enlargement and liver tumors in mice (Morimura et al., 2006). However, the implications of this process on hepatic lipid homeostasis remain unclear. Previous studies and our recent study indicated that WY-14643 (100 mg/kg) significantly promoted hepatomegaly (Woods et al., 2007; Gonzalez and Shah, 2008; Gyamfi and Wan, 2009; Fan et al., 2022). Thus, in the current study, mice were treated with 100 mg/kg WY-14643. The results showed that chronic treatment with WY-14643 induced significant hepatomegaly. Furthermore, two out of the six mice treated with WY-14643 developed visible liver tumors, and the expression levels of the liver tumor biomarkers AFP, KRT8, and GS were increased significantly in the WY-

14643-treated group, which indicated canceration of hepatocytes. Normally, GS is expressed in only one to three layers of cells around the CV area (Gebhardt and Mecke, 1983), but it was also expressed in hepatocytes around the PV area after chronic treatment with WY-14643. The results are consistent with the previously reported carcinogenic risk of chronic treatment with WY-14643 (Morimura et al., 2006). Similar to PPAR α , the nuclear receptors CAR and aryl hydrocarbon receptor (AhR) can also induce significant liver tumors after chronic activation (Dragani et al., 1985; Opitz et al., 2011). However, our recently published work demonstrated that chronic PXR activation did not induce or aggravate liver cancer in mice (Zhang et al., 2022). Distinct mechanisms between these nuclear receptors and hepatocarcinogenesis are needed further study.

Hepatomegaly is often attributed to hepatocyte enlargement and proliferation. Our previous studies found that the activation of some nuclear receptors, such as PPAR α , PXR, and CAR, can induce hepatomegaly, which were accompanied by hepatocyte enlargement and proliferation around the CV area the PV area, respectively (Jiao et al., 2020; Gao et al., 2021; Jiang et al., 2021; Zhao et al., 2021; Fan et al., 2022; Yao et al., 2022). Hepatocyte enlargement is mainly due to the accumulation of cellular contents, such as proteins, glycogen, or water, or the proliferation of subcellular organelles (Maronpot et al., 2010). Interestingly, the hepatocyte size was not significantly altered after chronic treatment with WY-14643 as determined by CTNNB1 staining. The liver tissue was obtained one week after the last treatment, during which hepatocyte size may have returned to the normal level. Therefore, these results may differ from samples collected 24 hours after treatment with WY-14643 for 10 consecutive days. Overexpression of proliferation-related proteins can evoke cell hyperproliferation, contributing to hepatomegaly, such as CDK4, PCNA, and myelocytomatosis oncogene (Peters et al., 1998). Consistently, CDK4 was upregulated in the WY-14643 group. CCND1, a cyclin, showed a rising tendency without significance. The PCNA staining showed that chronic treatment with WY-14643 induced hepatocyte proliferation around both the CV and PV areas, which was abolished in nontumoral livers. Thus, chronic treatment with WY-14643-induced hepatomegaly and liver tumors may be mainly attributed to enhanced hepatocyte proliferation.

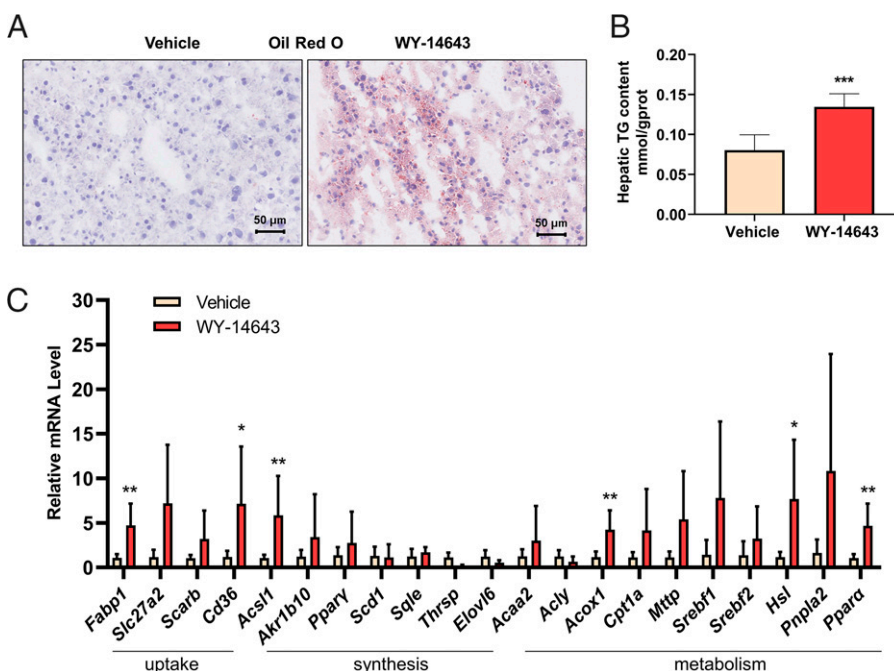


Fig. 5. Chronic treatment with WY-14643 induces hepatic TG accumulation by regulating the expression of TG homeostasis-related genes. (A) Representative pictures of oil red O staining in the vehicle and WY-14643 groups. Scale bar = 50 μ m. (B) Hepatic TG content ($n = 6$). (C) mRNA expression levels of genes related to TG homeostasis ($n = 6$). Data are presented as the means \pm S.D. Comparisons are based on the two-tailed Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the vehicle group.

ACOX1 and CYP4A are typical PPAR α downstream targets (Mandard et al., 2004). It has been reported that the expression levels of *Acox* and *Cyp4a* were significantly increased after 38 weeks of treatment with WY-14643 in both wild-type mice and PPAR α -humanized mice (Morimura et al., 2006). Consistently, in this study, the protein and mRNA expression levels of ACOX1 and CYP4A were also increased significantly in the WY-14643-treated group, indicating that PPAR α is continuously activated throughout the whole dosing period. In addition, the mRNA level of *Ppar α* was increased, but the protein expression level was not changed, which is in line with a previous study (Morimura et al., 2006) and suggests that chronic treatment with WY-14643 may induce PPAR α activation without affecting its expression. To investigate the potential crosstalk among different NRs, the expression levels of several NRs associated with liver enlargement were determined, which demonstrated that the expression levels of PXR and CAR were not changed, indicating that these two NRs may not be affected by chronic treatment with WY-14643. However, the expression level of FXR was increased significantly. A previous study showed that PPAR α activation can induce *Fxr* expression in fasted mice (Kok et al., 2003). During fasting, FXR activation can inhibit FA oxidation (Preidis et al., 2017). We speculate that the increase in FXR expression may be related to TG accumulation after chronic treatment with WY-14643. However, our previous study found that the expression level of FXR did not change after chronic PXR activation (Zhang et al., 2022). The specific relationship between PPAR α and FXR during this process needs further investigation.

As previously mentioned, lipid accumulation is conducive to hepatomegaly (Maronpot et al., 2010). PPAR α regulates the expression of FA oxidation-related enzymes in modulating lipid metabolism (Bougarne et al., 2018). However, the effects of chronic activation of PPAR α on hepatic lipids remain unclear. In the present study, lipidomic analysis and oil red O staining demonstrated that chronic treatment with WY-14643 mainly resulted in TGs accumulation in mouse livers. Hepatic TG metabolism is influenced by various factors. In our study, *Fabp1* and *Cd36*, related to FA uptake, were induced significantly by WY-14643 (Endemann et al., 1993; Podrez et al., 2000; Dickens et al., 2021). *Acs11*, involved in the synthesis of TGs (Li et al., 2009), was also elevated significantly by WY-14643. The upregulation of these lipogenic genes may contribute to TG accumulation in the liver. Of interest, *Acox1* and *Hsl*, two lipolysis genes, were also elevated. The changes in these lipogenic and lipolysis genes seem contradictory, but the final net effect was to promote TGs accumulation in the liver. Moreover, our previous study showed that short-term (5 days) and chronic (42 weeks) treatment with the specific PXR agonist PCN significantly induced TG accumulation (especially TGs) in mouse livers, contributing to hepatomegaly (Jiang et al., 2021; Zhang et al., 2022). Thus, TG accumulation might be a common effect on hepatic lipid homeostasis induced by PPAR α and PXR agonist treatment.

Lipids are essential for energy storage and body homeostasis. Lipids can be divided into eight categories, each of which can be subdivided into more varieties (Holčápek et al., 2018). In the current study, 923 ions were identified, and 43 potential hepatic lipid biomarkers were screened, which were mainly TGs. TGs can provide energy for hepatocyte proliferation involved in hepatomegaly and hepatocarcinogenesis (Santos and Schulze, 2012). The present study indicates that TGs accumulated after chronic treatment with WY-14643. We assume that WY-14643-promoted hepatocyte proliferation and liver enlargement occur partially via TG accumulation. Additionally, TG accumulation has been reported to be a central initiator of nonalcoholic fatty liver disease (Alves-Bezerra and Cohen, 2017). Thus, it may be necessary to be alert to the occurrence of hepatic steatosis during long-term treatment with PPAR α activators. These elevated TGs contained lower acyl carbon

numbers and double bonds. It was previously found that an increase of TGs content with low acyl carbon numbers and a double bond count is, in general, related to a clinically high risk for psychosis (Dickens et al., 2021).

In addition to TGs, many other lipids were significantly altered after chronic treatment with WY-14643. PI is an important component of cell membranes (Doering et al., 1990), and it has been reported to prevent liver injury via immune modulation (Inafuku et al., 2013). In our study, one PI was decreased significantly after chronic treatment with WY-14643, indicating the possibility that the immune system may be impaired during this process. Phosphatidylcholine (PC) and PE are the two most abundant phospholipids expressed on mammalian cell membranes (van der Veen et al., 2017). Abnormal PC/PE molar ratios can affect energy metabolism and the deposition of lipid droplets, which is related to many metabolic disorders (van der Veen et al., 2017). In the current study, two PEs were decreased significantly, which may influence the PC/PE molar ratio and metabolic homeostasis in the liver. PS is a class of anionic phospholipids synthesized from PC or PE (Kim et al., 2014). The decrease in PE may have led to the decrease in PS in our study. In addition, PS is a signal of cell apoptosis. When cells are apoptotic, PS will appear on the cell surface (Leventis and Grinstein, 2010). Thus, the decrease in PS may indicate a decrease in cell apoptosis and an increase in cell proliferation, which may lead to developing liver cancer.

To sum up, this study demonstrated that chronic treatment with WY-14643 induced liver enlargement and liver tumors in mice and disturbed lipid homeostasis by regulating genes involved in lipid metabolism. The accumulation of TG may promote the formation of liver tumors. Many clinical agents can stimulate PPAR α ; therefore, the risk of hepatic steatosis should be monitored when using PPAR α agonists chronically. These findings may help to further clarify the effects of sustained activation of PPAR α on liver lipid homeostasis and provide a basis for the clinically rational use of drugs that can activate PPAR α .

Authorship Contributions

Participated in research design: Fan, Gao, Huang, Bi

Conducted experiments: Yang, Zhang, Fan, Gao

Performed data analysis: Yang, Zhang, Fan, Gao

Wrote or contributed to the writing of the manuscript: Yang, Fan, Huang, Gao, Bi

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Address correspondence to: Huichang Bi, School of Pharmaceutical Sciences, Southern Medical University 1023# Shatai Nan Road, Baiyun District, Guangzhou 510515, China. E-mail: bihchang@smu.edu.cn; Yue Gao, School of Pharmaceutical Sciences, Sun Yat-Sen University 132# Waihuandong Road, Panyu District, Guangzhou 510006, China. E-mail: gaoy46@mail2.sysu.edu.cn; or Min Huang, School of Pharmaceutical Sciences, Sun Yat-Sen University 132# Waihuandong Road, Panyu District, Guangzhou 510006, China. E-mail: huangmin@mail.sysu.edu.cn.

Supplemental Data

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Chronic treatment with WY-14643 induces tumorigenesis and triglyceride accumulation in mouse livers

Jie Yang¹, Shicheng Fan¹, Yifei Zhang, Min Huang, Yue Gao, and Huichang Bi

Guangdong Provincial Key Laboratory of New Drug Design and Evaluation, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, China (J.Y., S.F., Y.Z., M.H., Y.G., H.B.)

NMPA Key Laboratory for Research and Evaluation of Drug Metabolism, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, Guangdong, China (S.F., H.B.)

¹Y.J. and S.F. contributed equally to this work.

Table S1. Lipids with fold-change > 3.0 and *VIP* > 1.0 after chronic treatment with WY-14643.

Lipid molecule	Formula	<i>m/z</i> (measured)	RT (min)	Ion type	Change trend ^a	Fold change	<i>P</i> value
TG(18:2/18:2/18:2)	C ₅₇ H ₉₈ O ₆	878.7363	14.7430	[M+NH ₄] ⁺	↑**	7.47	0.0022
TG(18:2/18:2/22:6)	C ₆₁ H ₉₈ O ₆	926.7363	14.4367	[M+NH ₄] ⁺	↑**	7.44	0.0022
TG(18:2/22:6/22:6)	C ₆₅ H ₉₈ O ₆	974.7363	14.1476	[M+NH ₄] ⁺	↑**	6.28	0.0022
TG(18:1/17:3/18:1)	C ₅₆ H ₉₈ O ₆	866.7363	15.5770	[M+NH ₄] ⁺	↑*	6.27	0.0260
TG(18:2/18:2/20:4)	C ₅₉ H ₉₈ O ₆	902.7363	14.6983	[M+NH ₄] ⁺	↑**	5.25	0.0022
TG(15:1/18:2/18:2)	C ₅₄ H ₉₄ O ₆	838.7050	15.1340	[M+NH ₄] ⁺	↑**	5.22	0.0022
TG(18:1/18:2/22:6)	C ₆₁ H ₁₀₀ O ₆	928.7520	14.8341	[M+NH ₄] ⁺	↑**	5.05	0.0022
TG(18:2/17:1/18:2)	C ₅₆ H ₉₈ O ₆	866.7363	14.9177	[M+NH ₄] ⁺	↑**	4.92	0.0022
TG(15:0/18:2/22:6)	C ₅₈ H ₉₆ O ₆	888.7207	14.4774	[M+NH ₄] ⁺	↑**	4.78	0.0087
TG(20:5/18:2/22:6)	C ₆₃ H ₉₆ O ₆	948.7207	14.4463	[M+NH ₄] ⁺	↑**	4.65	0.0022
TG(16:1/18:2/18:2)	C ₅₅ H ₉₆ O ₆	852.7207	14.6860	[M+NH ₄] ⁺	↑**	4.47	0.0022
TG(16:0/18:2/18:2)	C ₅₅ H ₉₈ O ₆	854.7363	15.1364	[M+NH ₄] ⁺	↑**	4.46	0.0022
TG(16:1/18:2/22:6)	C ₅₉ H ₉₆ O ₆	900.7207	14.3705	[M+NH ₄] ⁺	↑**	4.46	0.0022
TG(18:3/18:2/18:2)	C ₅₇ H ₉₆ O ₆	876.7207	14.3914	[M+NH ₄] ⁺	↑*	4.31	0.0260
TG(18:4/17:1/18:1)	C ₅₆ H ₉₆ O ₆	864.7207	15.1557	[M+NH ₄] ⁺	↑*	4.22	0.0152
TG(18:1/20:4/22:6)	C ₆₃ H ₁₀₀ O ₆	952.7520	14.5458	[M+NH ₄] ⁺	↑**	4.19	0.0022
TG(22:4/18:2/22:6)	C ₆₅ H ₁₀₂ O ₆	978.7676	14.6819	[M+NH ₄] ⁺	↑*	4.16	0.0152
TG(16:2/16:2/18:2)	C ₅₃ H ₉₀ O ₆	822.6737	14.0286	[M+NH ₄] ⁺	↑**	3.99	0.0022

TG(20:2/20:4/20:4)	C ₆₃ H ₁₀₂ O ₆	954.7676	14.9217	[M+NH ₄] ⁺	↑**	3.98	0.0043
TG(16:2/18:2/18:3)	C ₅₅ H ₉₂ O ₆	848.6894	14.0781	[M+NH ₄] ⁺	↑**	3.91	0.0022
TG(16:1/17:1/18:2)	C ₅₄ H ₉₆ O ₆	840.7207	14.8828	[M+NH ₄] ⁺	↑**	3.90	0.0022
TG(22:5/18:2/22:6)	C ₆₅ H ₁₀₀ O ₆	976.7520	14.3242	[M+NH ₄] ⁺	↑*	3.85	0.0389
TG(18:3/18:2/20:5)	C ₅₉ H ₉₄ O ₆	898.7050	14.0201	[M+NH ₄] ⁺	↑**	3.78	0.0022
TG(18:2/17:1/22:6)	C ₆₀ H ₉₈ O ₆	914.7363	14.4182	[M+NH ₄] ⁺	↑**	3.70	0.0022
TG(20:1/18:1/18:2)	C ₅₉ H ₁₀₆ O ₆	910.7989	16.0640	[M+NH ₄] ⁺	↑*	3.56	0.0260
TG(18:2/17:2/22:6)	C ₆₀ H ₉₆ O ₆	912.7207	14.2239	[M+NH ₄] ⁺	↑**	3.53	0.0043
TG(18:1/17:1/18:2)	C ₅₆ H ₁₀₀ O ₆	868.7520	15.3606	[M+NH ₄] ⁺	↑**	3.52	0.0022
TG(24:5/18:2/18:2)	C ₆₃ H ₁₀₄ O ₆	956.7833	15.2093	[M+NH ₄] ⁺	↑*	3.45	0.0411
TG(16:1/18:2/18:3)	C ₅₅ H ₉₄ O ₆	850.7050	14.3223	[M+NH ₄] ⁺	↑**	3.38	0.0022
TG(18:3/18:2/22:6)	C ₆₁ H ₉₆ O ₆	924.7207	14.1029	[M+NH ₄] ⁺	↑**	3.36	0.0043
TG(18:1/18:1/18:2)	C ₅₇ H ₁₀₂ O ₆	882.7676	15.5818	[M+NH ₄] ⁺	↑**	3.29	0.0087
TG(24:5/18:1/18:1)	C ₆₃ H ₁₀₈ O ₆	960.8146	15.8823	[M+NH ₄] ⁺	↑*	3.29	0.0152
TG(18:3/18:3/18:3)	C ₅₇ H ₉₂ O ₆	872.6894	13.9227	[M+NH ₄] ⁺	↑**	3.27	0.0022
TG(18:1/18:2/20:3)	C ₅₉ H ₁₀₂ O ₆	906.7676	15.2641	[M+NH ₄] ⁺	↑**	3.26	0.0022
TG(19:5/18:2/18:2)	C ₅₈ H ₉₄ O ₆	886.7050	14.1370	[M+NH ₄] ⁺	↑**	3.19	0.0087
TG(18:3/14:1/18:3)	C ₅₃ H ₈₈ O ₆	820.6581	13.7020	[M+NH ₄] ⁺	↑*	3.15	0.0495
TG(15:0/18:2/18:3)	C ₅₄ H ₉₄ O ₆	838.7050	14.5580	[M+NH ₄] ⁺	↑**	3.11	0.0022
TG(20:5/17:1/18:2)	C ₅₈ H ₉₆ O ₆	888.7207	14.9160	[M+H] ⁺	↑**	3.04	0.0022
TG(16:0/22:6/22:6)	C ₆₃ H ₉₈ O ₆	950.7363	14.3341	[M+NH ₄] ⁺	↑**	3.03	0.0022
PI(16:0/18:1)	C ₄₃ H ₈₁ O ₁₃ P ₁	836.5415	11.3429	[M-H] ⁻	↓*	0.3	0.03377

PE(16:0/16:1)	C ₃₇ H ₇₂ O ₈ N ₁ P ₁	689.4996	11.5684	[M+H] ⁺	↓***	0.29	0.0008
PE(16:1/20:5)	C ₄₁ H ₇₀ O ₈ N ₁ P ₁	735.4839	11.0324	[M+H] ⁺	↓**	0.28	0.0022
PS(16:0/19:0)	C ₄₁ H ₈₀ O ₁₀ N ₁ P ₁	777.5520	11.7312	[M-H] ⁻	↓*	0.21	0.0260

^a Comparisons are based on two-tailed Student's t test and Mann–Whitney U test. (* $P < 0.05$,

** $P < 0.01$, *** $P < 0.001$).

Table S2. Primer sequences for quantitative real-time PCR.

Gene	Species	Primer Sequence	
		Forward	Reverse
<i>Cyp4a10</i>	mouse	5'-TCCAGCAGTTCCCATCACCT-3'	5'-TTGCTTCCCCAGAACCATCT-3'
<i>Cyp4a14</i>	mouse	5'-TCAGTCTATTTCTGGTGCTGTTC-3'	5'-GAGCTCCTTGTCTTCAGATGGT-3'
<i>Acox1</i>	mouse	5'-TAACTTCCTCACTCGAAGCCA-3'	5'-AGTTCCATGACCCATCTCTGTC-3'
<i>Hsl</i>	mouse	5'-TGGCACACCATTTTGACCTG-3'	5'-TTGCGGTTAGAAGCCACATAG-3'
<i>Pnpla2</i>	mouse	5'-GGATGGCGGCATTTTCAGACA-3'	5'-CAAAGGGTTGGGTGTTTCAG-3'
<i>Cd36</i>	mouse	5'-ATGGGCTGTGATCGGAACTG-3'	5'-TTTGCCACGTCATCTGGGTTT-3'
<i>Ppara</i>	mouse	5'-AGAGCCCCATCTGTCCTCTC-3'	5'-ACTGGTAGTCTGCAAAACCAAA-3'
<i>Fabp1</i>	mouse	5'-ATGAACTTCTCCGGCAAGTACC-3'	5'-CTGACACCCCCTTGATGTCC-3'
<i>Slc27a2</i>	mouse	5'-TCCTCCAAGATGTGCGGTACT-3'	5'-TAGGTGAGCGTCTCGTCTCG-3'
<i>Scarb</i>	mouse	5'-AAACAGGGAAGATCGAGCCAG-3'	5'-GGTCTGACCAAGCTATCAGGTT-3'
<i>Acs11</i>	mouse	5'-TGCCAGAGCTGATTGACATTC-3'	5'-GGCATACCAGAAGGTGGTGAG-3'
<i>Akr1b10</i>	mouse	5'-CTAGTGCCAAACCAGAGGACC-3'	5'-TCCTGTATTGAGAAGGTGTCA-3'
<i>Pparγ</i>	mouse	5'-TATGGAGTGACATAGAGTGTGCT-3'	5'-CCACTTCAATCCACCCAGAAAG-3'
<i>Scd1</i>	mouse	5'-TTCTTGCGATACTCTGGTGC-3'	5'-CGGGATTGAATGTTCTTGTCGT-3'
<i>Sqle</i>	mouse	5'-ATAAGAAATGCGGGGATGTCAC-3'	5'-ATATCCGAGAAGGCAGCGAAC-3'
<i>Thrsp</i>	mouse	5'-ATGCAAGTGCTAACGAAACGC-3'	5'-CCTGCCATTCTCCCTTGG-3'
<i>Elovl6</i>	mouse	5'-GAAAAGCAGTTCAACGAGAACG-3'	5'-AGATGCCGACCACCAAAGATA-3'
<i>Acaa2</i>	mouse	5'-CTGCTACGAGGTGTGTTTCATC-3'	5'-AGCTCTGCATGACATTGCCC-3'
<i>Achy</i>	mouse	5'-CAGCCAAGGCAATTTTCAGAGC-3'	5'-CTCGACGTTTGATTAAGTGGTCT-3'

<i>Cpt1a</i>	mouse	5'-CTCCGCCTGAGCCATGAAG-3'	5'-CACCAGTGATGATGCCATTCT-3'
<i>Mtp</i>	mouse	5'-CTCTTGGCAGTGCTTTTTCTCT-3'	5'-GAGCTTGTATAGCCGCTCATT-3'
<i>Srebf1</i>	mouse	5'-GCAGCCACCATCTAGCCTG-3'	5'-CAGCAGTGAGTCTGCCTTGAT-3'
<i>Srebf2</i>	mouse	5'-GCAGCAACGGGACCATTCT-3'	5'-CCCCATGACTAAGTCCTTCAACT-3'
<i>18S</i>	mouse	5'-CCTGGATACCGCAGCTAGGA-3'	5'-GCGGCGCAATACGAATGCCCC-3'
