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Modulation of Lipid Metabolism by Celastrol

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Abstract

Hyperlipidemia, characterized by high serum lipids, is a risk factor for cardiovascular disease. Recent studies have identified an important role for celastrol, a proteasome inhibitor isolated from *Tripterygium wilfordii* Hook. f., in obesity-related metabolic disorders. However, the exact influences of celastrol on lipid metabolism remain largely unknown. Celastrol inhibited the terminal differentiation of 3T3-L1 adipocytes and decreased the levels of triglycerides in wild-type mice. Lipidomics analysis revealed that celastrol increased the metabolism of lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs), sphingomyelins (SMs), and phosphatidylethanolamines (PEs). Further, celastrol reversed the tyloxapol-induced hyperlipidemia induced associated with increased plasma LPCs, PCs, SMs, and ceramides (CMs). Among these lipids, LPC(16:0), LPC(18:1), PC(22:2/15:0), and SM(d18:1/22:0) were also decreased by celastrol in cultured 3T3-L1 adipocytes, mice, and tyloxapol-treated mice. The mRNAs encoded by hepatic genes associated with lipid synthesis and catabolism, including *Lpcat1*, *Pld1*, *Smpd3*, and *Sptc2*, were altered in tyloxapol-induced hyperlipidemia, and significantly recovered by celastrol treatment. The effect of celastrol on lipid metabolism was significantly reduced in *Fxr*-null mice, resulting in decreased *Cers6* and *Acer2* mRNAs compared to wild-type mice. These results establish that FXR was responsible in part for the effects of celastrol in controlling lipid metabolism and contributing to the recovery of aberrant lipid metabolism in obesity-related metabolic disorders.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://pubs.acs.org/doi/10.1021/acs.jproteome.8b00797) at DOI: 10.1021/acs.jproteome.8b00797. Histological and biochemical analysis; identification of LPC(16:0); tandem MS/MS of typical lipids; identification of LPC(18:1); primer sequences for qRT-PCR; changed lipids in three models (PDF)

The authors declare no competing financial interest.

Keywords

celastrol; lipidomics; hyperlipidemia; LC-MS

INTRODUCTION

Celastrol, also known as tripterine, is a natural triterpenoid compound isolated from *Tripterygium wilfordii* Hook. F.^{1,2} Celastrol has many pharmacological activities including anti-inflammatory, antioxidant, antiautoimmune, and anticancer effects.^{3–5} Celastrol can act as a potential therapeutic agent to counteract the symptoms of Alzheimer's disease.⁶ Earlier studies found that celastrol could not only inhibit the differentiation of adipocytes,⁷ but also increase the sensitivity of leptin⁸ and activate heat shock factor 1 (HSF1) to protect against obesity.⁹ Celastrol treatment significantly decreased high-fat diet (HFD)-induced intrahepatic triglyceride (TG) and nonalcoholic fatty liver disease (NAFLD) by activating sirtuin 1 (SIRT1).¹⁰ The prevalence of hyperlipidemia and lipid accumulation is increasing worldwide and is a major risk factor in the progression of cardiovascular and cerebrovascular diseases,¹¹ atherosclerosis, and fatty liver.¹² Therefore, celastrol may be a promising therapeutic agent to improve the pathological states that result from aberrant lipid metabolism, especially for obesity-related metabolic disorders.

Lipidomics has become a powerful tool to determine the changes in the abundance of various lipids in biofluids, tissues, and cells. Changes in individual lipids are found in various pathological states including hyperlipidemia,¹³ kidney disease,¹⁴ cerebrovascular diseases,¹⁵ and hepatocellular carcinoma.¹⁶ Lipidomics reveals a protective function for fatty acid oxidation in drug-induced hepatotoxicity, suggesting that this technique is an ideal method to obtain clues to celastrol's role in modulating lipid metabolism. In this study, three models, (1) 3T3-L1 adipocytes, (2) mice, and (3) tyloxapol-induced hyperlipidemia in mice, were used to evaluate the beneficial effects of celastrol on lipid metabolism and determine its influences on different lipid species under normal and induced pathological states. Lipidomic profiling revealed that celastrol promoted the metabolism of various classes of lipids including lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs), sphingomyelins (SMs), phosphatidylethanolamines (PEs), and ceramides (CMs). This study established a role for celastrol in lipid metabolism through regulating the mRNAs of genes involved in lipid biosynthesis and metabolism.

EXPERIMENTAL SECTION

Chemicals and Reagents

Celastrol (over 99.0% pure) was purchased from Chengdu Must Biotechnology (Chengdu, China). Tyloxapol and chenodeoxycholic acid (CDCA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). LPC (12:0) and other lipids standards were obtained from Avanti Polar Lipids (Alabaster, AL). Assay kits for aspartate transaminase (AST), aminotransferase (ALT), total cholesterol (TC), and TG were obtained from the Jiancheng Institute of Biotechnology (Nanjing, P.R. China). All other chemical reagents and solvents used were of analytic grade or above.

Animal Experiments

Male *Fxr*-null mice (6–8-week-old, C57BL/6J background) were transferred from the National Cancer Institute to the Kunming Institute of Botany, and male wide-type C57BL/6J (WT) mice (6–8-week-old) were purchased from the Slaccas Laboratory Animal Co., LTD (Hunan, China). *Fxr*-null and WT mice were cohoused in a temperature-controlled room, fed *ad libitum*, and maintained under a 12 h light/12 h dark cycle and humidity 50–60%. All mouse studies were performed in accordance with the guidelines of the National Institutes of Health, and all animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the Kunming Institute of Botany, Chinese Academy of Sciences. (I) Ten mice were randomly divided into two groups ($n = 5$), (1) control group (1% DMSO + 2% Tween 80 + 97% water) and (2) celastrol group (10 mg/kg body weight,⁸ gavage administration). The celastrol group was treated with celastrol (10 mg/kg) for 5 consecutive days. Whole blood was collected by orbital bleeding using capillary tubes 24 h after the last celastrol administration. Serum samples were isolated in tubes containing ethylenediaminetetraacetic acid dipotassium salt (EDTA-K2) after centrifugation at $2000 \times g$ for 5 min, which were stored at -80°C until use. (II) Fifteen WT mice were randomly divided into three groups, (1) control group (1% DMSO + 2% Tween 80 + 97% water, $n = 5$), (2) tyloxapol group (400 mg/kg body weight, gavage administration, $n = 5$), and (3) tyloxapol + celastrol group ($n = 5$). (III) Fifteen *Fxr*-null mice were randomly divided into three groups, (1) control group (1% DMSO + 2% Tween 80 + 97% water, $n = 5$), (2) tyloxapol group (400 mg/kg body weight,¹⁷ gavage administration, $n = 5$), and (3) tyloxapol + celastrol group ($n = 5$). Tyloxapol was dissolved in 0.9% saline. After celastrol treatment for 3 days, the tyloxapol group and the tyloxapol + celastrol group were given a single oral dose of tyloxapol (400 mg/kg) and the mice were killed 24 h after tyloxapol administration. Serum and liver were harvested and frozen at -80°C before analysis.

Cell Culture and Treatment

The 3T3-L1 adipocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Gibco, NY, USA) with 10% newborn calf serum (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere. 3T3-L1 adipocytes were seeded in a 6 cm culture plate at a density of 5×10^5 cells/well. The adipocytes were differentiated according to previous published methods with minor modification.¹⁸ Briefly, 2 days after reaching confluence (day 0), the cells were placed in DMEM supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin, 0.5

mM 3-isobutyl-1-methylxanthine (IBMX), 1 μ M dexamethasone, and 1 μ g/mL insulin. After 3 days (day 3), the medium was changed to DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1 μ g/mL insulin. After 1 day (day 4), the medium was replenished twice a day up to day eight. The cells were treated with celastrol according to a previous report, with minor modification.⁷ In brief, celastrol (0.4 μ M) was administered four times (days 0–3, 3–4, 4–6, and 6–8) ($n = 14$), and CDCA (100 μ M) was used as the positive control ($n = 14$).¹⁹ CDCA and celastrol were dissolved in DMEM with DMSO (concentration <0.05%). The cells were treated with DMEM containing equal DMSO as the control group ($n = 14$). After the termination of treatment, the culture wells were divided randomly into three groups for analysis of cell morphology, TG and TC measurement, and LC–MS analysis. For LC–MS analysis ($n = 6$), the adhered cells were washed twice with 5 mL of PBS after the medium from cell culture plates was aspirated. Then the cells were scraped using 1.0 mL of PBS and transferred to 1.5 mL tubes. Before lipids preparation for LC–MS analysis, the density of cells was normalized for each sample.^{20,21}

Sample Preparation for LC–MS Analysis

3T3-L1 cell samples for LC–MS analysis were prepared according to a previous report with minor modifications.^{22,23} The procedures were quickly performed on ice. Briefly, PBS was aspirated off from the cells after centrifugation ($600 \times g$, 4 °C, 5 min). The pellets were immediately extracted with a 800 μ L of chloroform and methanol (2:1) solution containing 5 μ M LPC(12:0) as an internal standards. After vortexing for 60 s, the samples were centrifuged at 13 000 rpm for 5 min, and the lower organic phase was collected and dried with N₂ gas at room temperature. Subsequently, the residue was dissolved in 25 μ L chloroform/methanol (1:1) solution, followed by diluting with 200 μ L of isopropanol/acetonitrile/H₂O (2:1:1). Three microliters of supernatant was injected to the UPLC–MS system for lipidomics profiling after centrifugation at $13\,000 \times g$ for 20 min. The serum samples used for lipidomics analysis were prepared according to the above method with minor changes as follows. Serum samples (25 μ L) were extracted with 100 μ L of cold chloroform and methanol (2:1) solution containing 5 μ M LPC(12:0) as internal standard. The extraction recovery of LPC(12:0) from plasma reached 95%. After vortexing for 30 s, the samples were allowed to stand for 5 min at room temperature. Other steps were similar to those used for lipid extractions from cultured cells.

UPLC-ESI-QTOFMS Analysis

An Agilent ultraperformance liquid chromatography/electrospray ion quadrupole time-of-flight mass spectrometer (UPLC-ESI-QTOFMS) (Agilent, Santa Clara, CA) was used for lipid proofing. The chromatographic conditions for serum lipidomics were as follows: XDB-C18 column (2.1×150 mm, 1.8 μ M); mobile phase, (A) water (containing 10 mM ammonium formate and 0.1% formic acid), and (B) acetonitrile/isopropanol (1:5, v/v) (containing 10 mM ammonium formate and 0.1% formic acid). The mobile phase was slightly changed according to earlier reports.^{24,25} The LC gradient used was as follows: 0–4 min, 61–81% B; 4–20 min, 81–90% B; 20–21 min, 90–100% B; and 21–24 min, 100–100% B; and the injection volume was 5 μ L. The flow rate was 0.3 mL/min, and the column temperature was maintained at 45 °C. The mass data were collected in positive ESI mode at

a capillary voltage of 3.5 kV, a nebulizer pressure of 35 psi, a drying gas and a collision gas flow of 9 L/min, a temperature of 350 °C, and a scan ranging from m/z 100 to 1000 Da.

Biochemical Assay and Histological Analysis

Serum AST and ALT activities were tested according to a method described in a previous report.²⁶ Total serum or 3T3-L1 cells TG and TC concentrations were measured using assay kits according to the manufacture's instruction (Jiancheng Bio-engineering Institute, Nanjing, China). Fresh liver tissues were fixed in 10% buffered formalin, embedded in paraffin and cut into five-micrometer serial sections, and stained with hematoxylin and eosin (HE). Histological examination was analyzed using light microscopy. Oil Red O staining was carried out as previously described.⁷

Gene Expression Analysis

Liver tissue (approximately 100 mg) was extracted using TRIzol reagent (Lifetechnologies, Carlsbad, CA). RT-QPCR was conducted on a CFX Connect Real-Time System (Bio-Rad Laboratories) using SYBR green PCR master mix (TaKaRa, Dalian, China). Primer sequences used are shown in Supplementary Table 1. QPCR conditions were 95 °C for 3 min; 95 °C for 10 s; 55 °C for 30 s; 72 °C for 40 s; 40 cycles. All targeted mRNA were normalized to the *Gapdh* mRNA. The relative mRNA expression levels were analyzed by the 2^{-CT} method.

Multivariate Data Analysis and Statistical Analysis

The acquired chromatographic and spectral data were processed by the Mass Profinder (MP) and Mass Profiler Professional (MPP) software (Agilent, Santa Clara, CA, USA), which generated a data matrix (containing peak areas, unique RT and m/z). Then all data were introduced to the SIMCA-P+13.0 software (Umetrics, Kinnelon, NJ) for PCA and OPLS-DA (p [CV-ANOVA] < 0.05). Different altered metabolites were displayed in the form of the score plot and loading plot. All lipids were matched by two databases, the Human Matabolome Database (HMDB, <http://www.hmdb.ca/>) and the Lipid Maps (<http://www.lipidmaps.org/>) with ppm < 10.0. Significance was determined as $VIP \geq 1$, $p(\text{corr})[1] < -0.8$ or $p(\text{corr})[1] > 0.8$, $P < 0.05$.

All data were expressed as the mean $\pm$ SEM. Differences among multiple groups were tested using one-way ANOVA followed by Dunnett's post hoc comparisons. Comparisons between 3T3-L1-C and 3T3-L1-Cel as well as Normal-C and Normal-Cel groups in two mice lines were tested by Student's t -test using SPSS 17.0 (IBM, Beijing, China). P -value of less than 0.05 was considered to be significant.

RESULTS

Celastrol Inhibits Adipocytes Differentiation

Compared to the vehicle-treated group, celastrol, similar to CDCA, significantly inhibited the differentiation of 3T3-L1 cells to adipocytes (Figure 1A). Lower cellular lipids were biochemically confirmed by measuring TG in 3T3-L1 adipocytes following celastrol exposure, which was in agreement with CDCA treatment (Figure 1B). Lipidomics was used

to further determine which lipids species were altered by celastrol. A score-plot of the PCA analysis ($R^2 = 0.93$ and $Q^2 = 0.86$) indicated that the celastrol group data set from analysis of 3T3-L1 cells were significantly separated from the control group (Figure 2A). In the loading *S*-plot of the OPLS-DA ($R^2 = 0.935$, $Q^2 = 0.992$, p [CV-ANOVA] < 0.0001), three decreased ions m/z 760.5863⁺ at 15.14 min, m/z 788.6173⁺ at 17.44 min, and m/z 786.5993⁺ at 15.96 min in 3T3-L1 adipocytes, were identified as PC(16:0/18:1), PC(18:0/18:1), and PC(18:1/18:1), respectively (Figure 2A). A total of 86 phospholipid species with VIP > 1 , $p(\text{corr})[1] < -0.8$ or $p(\text{corr})[1] > 0.8$, and $P < 0.05$ were detected, including 43 PCs, 10 SMs, 16 PEs, 2 CMs, and other lipid molecules (Table S-2). Most lipids were significantly reduced in 3T3-L1 adipocytes by celastrol exposure (Figure 3A–C). These results suggested that celastrol inhibited adipocyte differentiation.

Celastrol Promoted Lipid Metabolism in Normal Mice

The changes of lipids by celastrol were next examined in normal mice. After celastrol treatment for 5 days, the body weights of mice were significantly decreased compared to the vehicle-treated control group (Figure 1C). The TG levels in the celastrol-treated mouse livers were also lower than that of the control mouse livers (Figure 1D). However, no significant changes were observed in serum ALT and AST between the groups (Figure S-1A), indicating that the body weight decrease did not result from celastrol-induced toxicity (Figure S-1E). A score-plot from the PCA analysis ($R^2 = 0.79$) indicated that the celastrol group data set separated from the control group data set (Figure 2B). The change of lipids were then screened with $p(\text{corr})[1] < -0.8$ or $p(\text{corr})[1] > 0.8$, VIP > 1 , and $P < 0.05$ by comparing the celastrol-treated group with the control group. Three top decreased ions in the loading *S*-plot from the OPLS-DA ($R^2 = 0.94$, $Q^2 = 0.86$, p [CV-ANOVA] = 0.035), m/z 496.3393⁺ at 6.07 min, m/z 544.3143⁺ at 5.52 min, and m/z 784.5853⁺ at 14.10 min in the celastrol group, were identified as LPC(16:0), LPC(20:4) and PC(18:1/18:2), respectively (Figure 2B). The levels of a series of LPCs were significantly decreased following celastrol exposure (Figure 4A). In addition, celastrol reduced 16 PCs similar to that found with the celastrol-treated 3T3-L1 adipocytes (Figure 4A). Except for SM(d18:1/18:0) and SM(d18:1/18:1), the levels of other SMs were changed by celastrol (Figure 4B). There was no significant difference in Cer(d18:1/16:0) between the celastrol group and control group (Figure 4C). These results indicate that celastrol treatment can reduce the level of LPCs, PCs, and SMs in serum of normal mice. The detailed information about these lipids is shown in Table S-2. These results suggested that celastrol enhanced lipid metabolism in mice.

Tyloxapol-Induced Hyperlipidemia Was Recovered by Celastrol

It was demonstrated that tyloxapol administration dramatically disrupted lipid metabolism in mice, resulting in hyperlipidemia.²⁷ Here, the levels of TG and TC were increased in serum of tyloxapol-treated mice, indicating severe hyperlipidemia (Figure 1E). After celastrol treatment, the increased level of TG and TC in the tyloxapol group was reversed; however, no significant changes were observed in the level of liver TG and TC (Figure S-1B). Tyloxapol exposure significantly increased serum AST and ALT, the standard markers for liver damage (Figure S-1C), while histological analysis showed that tyloxapol did not induce obvious liver injury (Figure S-1D), suggesting that tyloxapol specifically resulted in hyperlipidemia independent of liver damage. Liver weights were not changed in the three

groups (Figure S-1F). A score-plot from the PCA analysis ($R^2 = 0.93$ and $Q^2 = 0.87$) revealed a significant separation between the tyloxapol group data set and the tyloxapol group treated with celastrol group data set (Figure 2C). In the scatter plot of PCA, the top changing ions were identified as PCs. A total of 63 phospholipid species were screened according to the rules that $p(\text{corr})[1] < -0.8$ or $p(\text{corr})[1] > 0.8$, $\text{VIP} > 1$, and $P < 0.05$ based on OPLS-DA ($R^2 = 0.999$, $Q^2 = 0.986$, $p[\text{CV-ANOVA}] < 0.0001$), including 33 PCs, 7 SMs, 3 CMs, and other lipid molecules. The detected lipids are shown in Table S-2. These lipids were significantly increased in hyperlipidemia induced by tyloxapol. As expected, celastrol significantly reversed the increased lipid levels in the tyloxapol group (Figure 5A–C). These results suggest that tyloxapol-induced hyperlipidemia was recovered by celastrol.

Celastrol Altered Expression of Hepatic Genes Involved in Lipid Biosynthesis and Metabolism Partly through the FXR

To determine the role of celastrol in mediating lipid metabolism, levels of mRNAs associated with lipid synthesis and metabolism were measured. Expression of three LPC-metabolism gene mRNAs, *Lpcat1*, *Lpcat2*, and *Lpcat3* (lysophosphatidylcholine acyltransferase 1, 2, and 3), was up-regulated in the tyloxapol group, and recovered by celastrol (Figure 6A). Although three LPC synthesis gene mRNAs, *Pla2g6*, *Pla2g7*, and *Pla2g12b* (phospholipase a2 g6, 7, and 12b), were not significantly changed in the tyloxapol group, celastrol treatment increased *Pla2g7* mRNA levels (Figure 6A). Two PC synthesis gene mRNAs, *Pcyt1b* encoding phosphate cytidyltransferase 1b, and *Chka* encoding hepatic choline kinase *a*, were elevated in the tyloxapol group and decreased by celastrol. *Pcyt1a* and *Chkb* mRNA levels were not different between the tyloxapol group and the tyloxapol group treated with celastrol (Figure 6B). Phospholipase D1 and D2 are involved in PC metabolism. Celastrol treatment increased *Pld1* mRNA (Figure 6B). The expression of SM and CM-related gene mRNAs, *Smpd1*, *Smpd2*, and *Smpd3*, encoding sphingomyelin phosphodiesterase 1, 2, and 3, respectively, were decreased in the tyloxapol group, and this decrease was recovered by celastrol treatment. Although the level of *Smpd4* mRNA was not changed in the tyloxapol group, celastrol still slightly increased its mRNAs (Figure 6C). The *Sgms1* mRNA encoding SM synthase 1 responsible for SM synthesis was increased in the tyloxapol group and lowered by celastrol.

It was reported that FXR regulates lipid homeostasis.^{28,29} Here, the elevated serum TG and TC levels in the tyloxapol group was slightly decreased (1.6-fold and 1.4-fold, respectively) by celastrol in *Fxr*-null mice (Figure 7A). By contrast, the levels of TG and TC in WT mice were decreased 3.8-fold and 1.7-fold by celastrol, indicating that celastrol regulated lipid metabolism in part through FXR. Lipidomics profiling revealed that celastrol may decrease the increased levels of CM in the tyloxapol group, although it elevated the levels of CM in 3T3-L1 adipocytes and WT mice. Celastrol could elevate the *Sptlc1* and *Sptlc2* mRNAs encoding serine palmitoyltransferase long chain base subunit 1 and 2, respectively, that are responsible for CM synthesis. *Sptlc2* mRNA was decreased in the tyloxapol group in WT mice (Figure 7B). CM-related metabolism gene mRNAs, including alkaline ceramidase 2 (*Acer2*) and ceramide alkaline (*Cerk*), were upregulated in WT mice and *Fxr*-null mice compared with mRNA levels detected in tyloxapol group (Figure 7B,C). In addition, other CM synthesis and metabolism gene mRNAs, *Lass1*, encoding homologue ceramide synthase

1, *Cers4* and 5 encoding ceramide synthase 4 and 5, respectively, *Degs1* encoding degenerative spermatocyte homologue 1, alkaline ceramidase 3 (*Acer3*) were not significantly changed in the tyloxapol group and after celastrol treatment in WT mice (Figure 7B,C). Compared to WT mice, the increased levels of *Acer2* and *Cers6* mRNAs encoding ceramide synthase 6 after celastrol treatment were lower in *Fxr*-null mice. These results suggest that celastrol exposure markedly restored the aberrant lipid metabolism-induced by tyloxapol and that FXR plays a role in the mechanism of celastrol action.

Comparison of Lipids Changes in Three Models by Celastrol Exposure

To further determine the common lipids regulated by celastrol, all lipids in the three models-treated with celastrol were used to prepare a Venn diagram (Figure 8A). A total of 75, 49, and 57 lipids were significantly changed in 3T3-L1 adipocytes, normal mice, and tyloxapol-induced hyperlipidemia by celastrol, respectively. Among these changed lipids, four lipids, LPC(16:0), LPC(18:1), PC(22:2/15:0), and SM(d18:1/22:0), were collectively decreased in the three models by celastrol (Figure 8B–E, Table 1). Another four lipids were reduced in both the 3T3-L1 adipocytes and normal mouse groups, while no obvious changes were observed in the tyloxapol model. Twelve lipids were decreased in both 3T3-L1 adipocytes and the tyloxapol model, while no changes observed in mice-treated with celastrol. Moreover, 13 lipids were collectively changed in both mice and tyloxapol model, but there were no significant changes in 3T3-L1 adipocytes following celastrol treatment (Figure 8A, Table 1).

Identification of Lipids That Changed in Three Models

To confirm the identity of altered lipids, their retention times and MS/MS fragmentation patterns were compared with authentic standards and public databases, including HMDB and the lipid maps. The LPCs showed similar fragmentation patterns, and the characteristic fragments included m/z 184 [$C_5H_{15}NO_4P$]⁺ and m/z 104 [$C_5H_{14}NO$]⁺. MS/MS fragments of two typical lipids LPC(16:0) (RT = 6.07 min) and LPC(18:1) (RT = 6.34 min) were confirmed by comparing the sample with an standard (Figures S-2 and S-3). Similarly, m/z 140 [$C_2H_7O_4NP$][−] is a characteristic fragment of PEs. PE (18:0/18:1) was identified on the basis of its fragmentation pattern, as revealed by the presence of m/z 140 [$C_2H_7O_4NP$][−], m/z 281 [$C_{17}H_{33}COO$][−], and m/z 283 [$C_{17}H_{35}COO$][−].³⁰ The m/z 784.5885⁺, 703.5750⁺, 313.2751⁺, 887.5644⁺, and 744.5553⁺ ions were identified as PC(18:1/18:2), SM(d18:1/16:0), MG(16:0/0:0/0:0), PI(20:4/18:0), and PE(18:0/18:1), respectively (Figure S-4A–E). Consistent with the characteristic MS/MS fragments found in previous reports,^{22,24,30–32} SMs, CMs, PCs, and PEs were identified in the present study.

DISCUSSION

Celastrol repressed adipocytes differentiation, decreased lipids in normal mice, and recovered tyloxapol-induced hyperlipidemia. Metabolic disorders of lipid metabolism are associated with diseases such as diabetes,³³ NAFLD,²⁴ and prostate cancer.³⁴ In this study, by use of a lipidomics approach in cells and mouse serum, the levels of PCs, LPCs, SMs, and CMs were significantly altered after celastrol administration. Tyloxapol-induced hyperlipidemia elevated PCs, LPCs, SMs, and CMs, which are similar to HFD-induced

hyperlipidemia.³⁵ More importantly, the metabolic disorders resulting from lipids induced by tyloxapol were reversed after celastrol treatment through regulating lipid biosynthesis- and metabolism-related genes. These results confirmed that celastrol can significantly influence lipid metabolism, as revealed by analysis of lipidomics, biochemistry, and histology.

Phospholipids compounds are the major components of cell membranes including PCs, PEs, and PIs. In this study, the levels of PCs and PEs were altered by celastrol in the three models. LPCs are important signaling molecules that are involved in several diseases including cholestasis,³⁶ atherosclerosis,³⁷ acute colitis,³⁸ and obesity.³² Celastrol alleviated DSS-induced colitis through the regulation of LPCs and SMs metabolism.³⁹ Moreover, SM(d18:1/22:0) was reported as a potential biomarker for the diagnosis of hepatic steatosis, as serum SM(d18:1/22:0) is correlated with steatosis associated with chronic HCV infection.⁴⁰ Here, SM(d18:1/22:0) was decreased 50% by celastrol in all three models. The expression of genes related to SMs metabolism, including *Smpd1*, *Smpd2*, and *Smpd3*, as revealed by mRNA analysis, was inhibited in the tyloxapol model. The mRNAs levels could be elevated by celastrol, suggesting that the decrease of SMs contributes to the improvement of the aberrant lipid accumulation.

Another intriguing finding was that celastrol reduced the elevated CMs induced by tyloxapol. Previous studies showed that CMs regulate energy production and nutrient utilization⁴¹ and are involved in the development of obesity, dyslipidemia,⁴² and NAFLD.⁴³ CMs derived from the intestinal FXR induction of *Smpd3* causes increased hepatic steatosis as a result of an elevation in SREBP-1c signaling and the resultant increased fatty acid synthesis.^{24,44} Inhibition of intestinal FXR could decrease intestinal and serum ceramide levels through suppression of the expression of genes such as *Smpd3* involved in CM synthesis and that ceramide serves as a biochemical conduit between the gut and liver during the pathogenesis of fatty liver.⁴⁴ Glycine- β -MCA (Gly-MCA), an intestinal FXR antagonist, improved obesity and fatty liver induced by HFD and promoted adipose browning leading to increased thermogenesis.⁴⁵ The FXR agonist GW4064 also alleviated hepatic inflammation induced by endotoxin⁴⁶ and insulin resistance that results from HFD feeding.⁴⁷ The present study revealed that celastrol could decrease adipocyte differentiation and TG levels in cultured 3T3-L1 adipocytes. Although its effect was similar to that of CDCA, further studies are needed to examine the mechanism by which celastrol modulates FXR signaling.

The levels of *Sptlc1* and *Sptlc2* mRNAs, encoding enzymes involved in the synthesis of CMs in liver, were decreased in the tyloxapol group, and these changes in gene expression restored by celastrol treatment. In both *Fxr*-null and WT mice, the *Acer2* and *Cerk* mRNAs, involved in the metabolism of CMs, were elevated by celastrol. It was reported that PCs and CMs are converted to SMs and DAGs by SGMS1;⁴⁸ *Sgms1* mRNAs was decreased by celastrol. Additionally, serine palmitoyltransferase (SPT) plays an important role in phospholipids biosynthesis. A previous study revealed that liver-specific *Sptlc2*-deficient mice lost hepatocyte polarity and tumorigenesis.⁴⁹ The present study found that celastrol could recover the elevated PCs and SMs induced by tyloxapol. These data showed that celastrol may maintain the homeostasis of lipid metabolism by regulating genes involved in lipid synthesis and catabolism, which was dependent in part on FXR signaling.

In conclusion, the present study revealed that celastrol improved tyloxapol-induced hyperlipidemia in mice and suppressed adipocytes differentiation in 3T3-L1 adipocytes using lipidomics approach. The results indicated that celastrol contributed to the homeostasis of lipids in the body via the regulation of the genes associated with lipids biosynthesis and metabolism and that FXR signaling might be involved in this process. Thus, celastrol could be a promising candidate to repair the aberrant lipid metabolism in obesity-related metabolic disorders.

Supplementary Material

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Figure 1. Histological examination and biochemical analysis. (A) Oil Red O staining in 3T3-L1 adipocytes. Cells were treated with celastrol (0.4 μ M), and CDCA (100 μ M) was used as a positive control. (B) Level of 3T3-L1 adipocytes TG. (C) Body weight in normal mice. (D) Level of liver TG in normal mice. * P < 0.05, ** P < 0.01, and *** P < 0.001 versus control group. (E) Serum TG and TC levels in control, tyloxapol, and tyloxapol+celastrol groups. All data were expressed as the mean $\pm$ SEM *** P < 0.001 for control group versus tyloxapol group; # P < 0.05, and ### P < 0.001 for tyloxapol +celastrol group versus tyloxapol group.

Figure 2.

PCA and OPLS-DA analysis of three models. (A) PCA score plot and S-plot from OPLS-DA (p [CV-ANOVA] < 0.0001) derived from UPLC-QTOFMS data of 3T3-L1 adipocytes. (B) PCA score plot and S-plot from OPLS-DA (p [CV-ANOVA] = 0.035) derived from UPLC-QTOFMS data of serum ions in normal mice. (C) PCA score plot and loading plot derived from UPLC-QTOFMS data of serum ions in tyloxapol model.

Figure 3.

Celastrol affected the level of lipids in 3T3-L1 cells. (A) Heatmap analysis of the changed lipid in 3T3-L1 cells. (B) Celastrol decreased the level of SMs. (C) CMs levels were increased in 3T3-L1 cells. The metabolites abundance was decreased with the color changing from red to green. The levels of the metabolites were expressed as the fold change by comparing with control group. All data were expressed as the mean $\pm$ SEM ** P < 0.01 and *** P < 0.001 for celastrol group versus control group.

Figure 4.

Serum lipidomics analysis revealed that celastrol improved lipid metabolism in normal mice. (A) Heatmap analysis of the changed lipid in normal mice. (B) Celastrol decreased the levels of SMs. (C) Level of CMs was not obviously changed by celastrol in normal mice. The metabolites abundance was decreased with the color changing from red to green. The levels of the metabolites were expressed as the fold change by comparing with control group. All data were expressed as the mean $\pm$ SEM * P < 0.05 and *** P < 0.001 for control group versus celastrol group.

Figure 5.

Celastrol recovered aberrant lipid metabolism in tyloxapol model. (A) Heatmap analysis of the recovered lipid induced by tyloxapol. Celastrol decreased the levels of (B) SMs and (C) CMs. The metabolites abundance was decreased with the color changing from red to green. The levels of the metabolites were expressed as the fold change by comparing with control group. All data were expressed as the mean $\pm$ SEM * P < 0.05 and *** P < 0.001 for tyloxapol group versus control group; # P < 0.05 and ## P < 0.01 for tyloxapol group versus tyloxapol+celastrol group.

Figure 6. QPCR analysis of the gene expression of lipids metabolism and biosynthesis in tyloxapol model. (A) LPC synthesis- and metabolism-related genes. (B) PC synthesis- and metabolism-related genes. (C) SM synthesis- and metabolism-related genes. All targeted mRNA was normalized to the *Gapdh* mRNA as internal control. All data were expressed as the mean $\pm$ SEM * P < 0.05, ** P < 0.01, and *** P < 0.001 for control mice versus tyloxapol-treated mice; # P < 0.05, ## P < 0.01, and ### P < 0.001 for tyloxapol+celastrol-treated mice versus tyloxapol-treated mice.

Figure 7.

Role of FXR in celastrol modulating lipid metabolism. (A) Serum TG and TC levels in control, tyloxapol, and tyloxapol+celastrol groups in *Fxr*-null mice. (B) CM related genes in WT mice. (C) CM related genes in *Fxr*-null mice. All data were expressed as the mean $\pm$ SEM * P < 0.05 and ** P < 0.01 for control mice versus tyloxapol-treated mice; # P < 0.05, ## P < 0.01, and ### P < 0.001 for tyloxapol+celastrol-treated mice versus tyloxapol-treated mice.

Figure 8.

Comparison of metabolites changed in three models. (A) Venn diagram of the three lipid models. (B) LPC(16:0), (C) LPC(18:1), (D) PC(22:2/15:0), and (E) SM(d18:1/22:0). All data were expressed as the mean $\pm$ SEM * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 1.
Changed Metabolites in 3T3-L1 Cell, WT Mice, and Tyloxapol Model after Celastrol Exposure

no.	RT (min)	[M + H] ⁺ m/z	error (ppm)	identity	empirical formula	trend induced by celastrol ^a		
						3T3-L1 cell	WT mice	tyloxapol model
Changes in Three Models								
1	6.34	522.3553	0.40	LPC(18:1)	C ₂₆ H ₅₂ NO ₇ P	↓	↓	↓
2	6.07	496.3393	-0.81	LPC(16:0)	C ₂₄ H ₅₀ NO ₇ P	↓	↓	↓
3	17.05	800.6163	0.00	PC(22:2/15:0)	C ₄₅ H ₈₆ NO ₈ P	↓	↓	↓
4	20.19	787.6693	0.76	SM(d18:1/22:0)	C ₄₅ H ₉₁ N ₂ O ₆ P	↓	↓	↓
Changes in 3T3-L1 Cell and Mice								
1	15.29	810.5993	-0.99	PC(18:0/20:4)	C ₄₆ H ₈₄ NO ₈ P	↓	↓	-
2	14.10	784.5853	0.25	PC(18:1/18:2)	C ₄₄ H ₈₂ NO ₈ P	↓	↓	-
3	22.33	815.6993	-0.86	SM(d18:1/24:0)	C ₄₇ H ₉₅ N ₂ O ₆ P	↓	↓	-
4	19.71	813.6863	2.33	SM(d18:1/24:1)	C ₄₇ H ₉₃ N ₂ O ₆ P	↓	↓	-
Changes in 3T3-L1 Cell and Tyloxapol Model								
1	12.54	780.5553	1.92	PC(18:2/18:3)	C ₄₄ H ₇₈ NO ₈ P	↓	-	↓
2	12.81	806.5693	-0.12	PC(18:2/20:4)	C ₄₆ H ₈₀ NO ₈ P	↓	-	↓
3	13.21	782.5683	-1.4	PC(18:1/18:3)	C ₄₄ H ₈₀ NO ₈ P	↓	-	↓
4	13.65	782.5683	-1.4	PC(16:0/20:4)	C ₄₄ H ₈₀ NO ₈ P	↓	-	↓
5	14.40	808.5853	0.25	PC(18:0/20:5)	C ₄₆ H ₈₂ NO ₈ P	↓	-	↓
6	14.50	772.5813	-4.91	PC(20:2/15:0)	C ₄₃ H ₈₂ NO ₈ P	↓	-	↓
7	15.14	760.5863	-1.57	PC(16:0/18:1)	C ₄₂ H ₈₂ NO ₈ P	↓	-	↓
8	17.44	788.6173	1.21	PC(18:0/18:1)	C ₄₄ H ₈₆ NO ₈ P	↓	-	↓
9	15.86	836.6173	1.14	PC(18:0/22:5)	C ₄₈ H ₈₆ NO ₈ P	↓	-	↓
10	16.49	812.6143	-2.52	PC(18:0/20:3)	C ₄₆ H ₈₆ NO ₈ P	↓	-	↓
11	14.67	766.5733	-1.55	PC(P-16:0/20:4)	C ₄₄ H ₈₀ NO ₇ P	↓	-	↓
12	11.24	608.4653	0.6	LPC(24:0)	C ₃₂ H ₆₆ NO ₇ P	↓	-	↓
Changes in Mice and Tyloxapol Model								
1	14.81	734.5703	1.22	PC(16:0/16:0)	C ₄₀ H ₈₀ NO ₈ P	-	↓	↓

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no.	RT (min)	[M + H] ⁺ m/z	error (ppm)	identity	empirical formula	trend induced by celastrol ^a		
						3T3-L1 cell	WT mice	tyloxapol model
2	15.84	860.6143	-2.38	PC(20:1/22:6)	C ₅₀ H ₈₆ NO ₈ P	-	↓	↓
3	15.96	786.5993	-1.78	PC(18:1/18:1)	C ₄₄ H ₈₄ NO ₈ P	-	↓	↓
4	18.67	814.6323	0.37	PC(18:2/20:0)	C ₄₆ H ₈₈ NO ₈ P	-	↓	↓
5	13.15	744.5523	-1.95	PC(18:2/15:0)	C ₄₁ H ₇₈ NO ₈ P	-	↓	↓
6	14.58	746.5673	2.81	PC(18:1/15:0)	C ₄₁ H ₈₀ NO ₈ P	-	↓	↓
7	13.62	887.5623	-2.34	PI(20:4/18:0)	C ₄₇ H ₈₃ O ₁₃ P	-	↓	↓
8	5.56	520.3413	2.98	LPC(18:2)	C ₂₆ H ₅₀ NO ₇ P	-	↓	↓
9	4.98	518.3243	0.39	LPC(18:3)	C ₂₆ H ₄₈ NO ₇ P	-	↓	↓
10	5.78	546.3543	2.00	LPC(20:3)	C ₂₈ H ₅₂ NO ₇ P	-	↓	↓
11	5.52	544.3413	2.85	LPC(20:4)	C ₂₈ H ₅₀ NO ₇ P	-	↓	↓
12	5.79	570.3543	-1.92	LPC(22:5)	C ₃₀ H ₅₂ NO ₇ P	-	↓	↓
13	5.17	494.3243	0.41	LPC(16:1)	C ₂₄ H ₄₈ NO ₇ P	-	↓	↓

^a↓, decreased; -, no significant differences.