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In Brief
The present study used metabolomics, transcriptomics, protein analysis by immunoblots, chemical inhibition and gene knockout mice to determine the effects of celastrol on cholestasis and its mechanisms. Samples from patients were used to validate our findings, and the data supported the conclusions that celastrol protected against cholestatic liver injury through modulation of the SIRT1 and FXR.

Highlights
- 1. Celastrol alleviated cholestatic liver injury.
- 2. Celastrol inhibited the decrease of SIRT1 induced by deoxycholic acid.
- 3. SIRT1-FXR signaling pathway mediated the effect of celastrol.
Celastrol Protects From Cholestatic Liver Injury Through Modulation of SIRT1-FXR Signaling*

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Celastrol, derived from the roots of the Tripterygium Wilfordi, shows a striking effect on obesity. In the present study, the role of celastrol in cholestasis was investigated using metabolomics and transcriptomics. Celastrol treatment significantly alleviated cholestatic liver injury in mice induced by α-naphthyl isothiocyanate (ANIT) and thioacetamide (TAA). Celastrol was found to activate sirtuin 1 (SIRT1), increase farnesoid X receptor (FXR) signaling and inhibit nuclear factor-kappa B and P53 signaling. The protective role of celastrol in cholestatic liver injury was diminished in mice on co-administration of SIRT1 inhibitors.

Further, the effects of celastrol on cholestatic liver injury were dramatically decreased in FXR-null mice, suggesting that the SIRT1-FXR signaling pathway mediates the protective effects of celastrol. These observations demonstrated a novel role for celastrol in protecting against cholestatic liver injury through modulation of the SIRT1 and FXR. Molecular & Cellular Proteomics 18: 520–533, 2019. DOI: 10.1074/mcp.RA118.000817.

Cholestasis, including primary biliary cirrhosis and primary sclerosing cholangitis, causes intracellular accumulation of toxic bile acids (BAs) (1). If left untreated, cholestasis may lead to liver fibrosis, cirrhosis, and even liver failure. Standards of care for cholestatic liver disease are currently limited to the use of ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) approved by U.S. Food and Drug Administration for slowing the progression of primary biliary cirrhosis. Because UDCA and OCA may activate farnesoid X receptor (FXR), both drugs protect against cholestasis mainly through the regulation of the BA metabolism and transport. However, both UDCA and OCA have no therapeutic effect for primary sclerosing cholangitis and other adult cholestatic disorders (2). Additionally, ~50% patients show no response for the treatment with UDCA, and OCA treatment shows the limited follow-up data in clinical case survey (3). Some other therapeutic strategies are screened for the treatment of cholestasis, for example, the activation of peroxisome proliferator-activated receptor α (PPARα) shows the protective effects on cholestasis (4). Thus, developing new drugs and looking for new targets for the treatment of cholestatic liver disease are necessary.

Mass spectrometry-based metabolomics has become a powerful tool for the characterization of metabolites in biological matrices and has been applied to studies in hepatology. Notably, metabolomic data for nonalcoholic steatohepatitis (5), cholestasis (6), viral hepatitis (7), liver dysfunction (8, 9), and hepatocellular carcinoma (10) have been obtained in the recent years. These studies revealed that metabolic disorders involving BAs and phospholipids are a core phenotype for hepatobiliary diseases (5, 10, 11). Additionally, metabolomics was used to determine the role of nuclear receptors including PPARs, FXR, and pregnane X receptor in drug pharmacology and toxicology (12, 13). Metabolomics has become an important tool to probe for disease mechanism that can lead to new therapeutics target for hepatobiliary diseases.

In the current study, mass spectrometry-based metabolomics was adopted to determine the changes of metabolites in patients with cholestatic liver disease and cholestasis mouse models. Among these BAs increased in cholestasis, deoxycholic acid (DCA), taurocholic acid (TCA), and taurodeoxycholic acid (TDCA) decreased the level of sirtuin 1 (SIRT1) in primary hepatocytes. Celastrol, a regulator of SIRT1, could elevate the expression of SIRT1 in primary hepatocytes, and alleviate cholestatic liver injury-induced by α-naphthyl isothio-
cyanate (ANIT) and thioacetamide (TAA). SIRT1 inhibition by EX527 and salermide, and Fxr-null mice were employed to explore the mechanism by which celastrol protects against cholestatic liver injury. This study demonstrates that celastrol improves cholestasis and recovers the change of BAs through the activation of SIRT1-FXR signaling.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Statistical Rationale**—The experimental design and statistical rationale for each of the experiments conducted in this work have been described in each subsection. RNAseq analysis was carried out based on produced biological triplicates. QPCR analysis was carried out using four to six biological and three technological replicates, including no template negative controls.

**Chemicals and Reagents**—Celastrol was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cholic acid (CA), UDCA, hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurohydroxydeoxycholic acid (THDCA), taurochenodeoxycholic acid (TCDDCA), TDCA, tauroliothocholic acid (TLCA), glycochenodeoxycholic acid (GCDDCA), dehydrocholic acid (DHC), and glycolithocholic acid (GLCA) were purchased from Sigma-Aldrich (St. Louis, MI). Tauro-β-muricholic acid (TβMCA), ω-muricholic acid (ωMCA), and hyocholic acid (HCA) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). EX527, salermide, SRT1720, and tauroursodeoxycholic acid (TUDCA) were purchased from Medchemexpress (Monmouth Junction, NJ). Tauro-α-muricholic acid (TαMCA) and β-muricholic acid (βMCA) were purchased from Steraloids (Newport, RI).

**Patients**—Twelve patients with cholestasis and eighteen healthy volunteers aged from 33 to 62 years were recruited from the Second Affiliated Hospital of Kunming Medical University in this study. All volunteers were evaluated by clinicians before and during the study to ensure they remained in good health. The patients with cholestasis were diagnosed by clinicians in this study, and the patients information is summarized in Table I. Written informed consents were obtained from each participant included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution’s human research committee (the Second Affiliated Hospital of Kunming Medical University). Blood was collected before the first administration of therapeutic drugs. Blood was collected in ethylenediaminetetraacetic acid tubes, which was left on ice immediately. Then, samples were centrifuged at 2000 × g for 5 min at 4 °C. Serum samples were transferred to new tubes (1.5 ml) and immediately stored at −80 °C until use. Physiological serum parameters of clinical serum samples were measured using the VetScan VS2 Comprehensive Diagnostic Profile (Abaxis, Inc., Union City, CA). The human serum samples collected here were used to discover the biomarkers associated with cholestasis.

**Animals**—Male 6-week-old Fxr-null mice (C57BL/6J background) were previously described (14) and male 6-week-old wide-type C57BL/6J (WT) mice were purchased from the Slaccas Laboratory Animal Co., LTD (Hunan, China). Fxr-null and WT mice were housed on a 12-hour dark/light cycle with standard rodent chow and water ad libitum to minimize potential microbiome effects. All animal experiments were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Botany, Chinese Academy of Sciences.

**Experiment 1**—To find the common increased ions in ANIT- and TAA-induced cholestasis, WT mice were randomly assigned into four groups (n = 6): (1) ANIT-Control; (2) ANIT; (3) TAA-Control; (4) TAA. ANIT group was given a single oral dose of ANIT (75 mg/kg dissolved in corn oil) (19), and TAA group was given a single intraperitoneal dose of TAA (300 mg/kg dissolved in physiologic saline solution) (16).

**Experiment 2**—To investigate the protective effect of celastrol on ANIT-induced cholestasis, the WT (n = 6) and Fxr-null (n = 5) mice were randomly assigned into three groups, respectively: (1) control; (2) ANIT; (3) ANIT + Celastrol. ANIT + Celastrol group was orally treated with celastrol (10 mg/kg dissolved in 1% DMSO + 2% Tween 80 + 97% water) for 5 consecutive days (17). After celastrol was treated for 3 days, ANIT and ANIT + Celastrol groups were given a single oral dose of ANIT.

**Experiment 3**—To investigate the protective effect of celastrol on TAA-induced cholestasis, the WT (n = 6) and Fxr-null (n = 6) mice were randomly assigned into three groups, respectively: (1) control; (2) TAA; (3) TAA + Celastrol. TAA + Celastrol group was orally treated with celastrol for 5 consecutive days. After celastrol treatment for 4 days, the TAA and TAA + Celastrol groups mice were given a single intraperitoneal dose of TAA.

**Experiment 4**—To determine the influence of SIRT1 in the protective effect of celastrol on ANIT-induced cholestasis, co-treatment with SIRT1 inhibitors EX527 (n = 4) and salermide (n = 5) were used, respectively. For the EX527 experiment, mice were randomly assigned into six groups: (1) control; (2) ANIT; (3) ANIT + Celastrol; (4) ANIT + Celastrol + EX527; (5) EX527; (6) ANIT + EX527. For salermide experiment, the mice were randomly assigned into four groups: (1) control; (2) ANIT; (3) ANIT + Celastrol; (4) ANIT + Celastrol + salermide. ANIT + Celastrol + EX527/salermide group was co-treated with EX527 (10 mg/kg dissolved in corn oil, intraperitoneal administration, 30 min before celastrol) or salermide (30 mg/kg dissolved in DMSO, intraperitoneal administration, 30 min before celastrol) (18) or salermide (30 mg/kg dissolved in DMSO, intraperitoneal administration, 30 min before celastrol) (19) and celastrol for five consecutive days.

**Experiment 5**—To investigate the role of SIRT1 on the protective effect of celastrol in TAA-induced cholestasis, EX527 (n = 4), salermide (n = 5), and SRT1720 (n = 5) were used. For EX527 experiment, the mice were randomly assigned into six groups: (1) control; (2) TAA; (3) TAA + Celastrol; (4) TAA + Celastrol + EX527; (5) EX527; (6) TAA + EX527. For salermide experiment, the mice were randomly assigned into four groups: (1) control; (2) TAA; (3) TAA + Celastrol; (4) TAA + Celastrol + salermide. ANIT + Celastrol + EX527/salermide group was co-treated with EX527 (10 mg/kg dissolved in corn oil, intraperitoneal administration, 30 min before celastrol) (18) or salermide (30 mg/kg dissolved in DMSO, intraperitoneal administration, 30 min before celastrol) (19) and celastrol for five consecutive days.
Activation of SIRT1-FXR Improves Cholestasis

All mice were killed 48 h after ANIT administration and 24 h after TAA administration. Serum and liver samples were harvested and frozen at −80 °C before analysis.

**UPLC-ESI-QTOFMS-based Metabolomic Analysis**—The preparation of serum and liver samples and the metabolomics analysis was described previously (4, 20). The liquid chromatography system consisted of a 1290 Autosampler, Quat Pump, and Photodiode Array Detector (Agilent, Santa Clara, CA). The endogenous metabolites were separated via an XDB-C18 column (2.1 mm × 100 mm, 1.8 μm). The mobile phase comprised 0.01% aqueous formic acid and acetonitrile containing 0.01% formic acid. The flow rate was 0.3 ml/min with a gradient ranging from 2% to 98% acetonitrile in 16 min run. The column temperature was maintained at 45 °C through the run. The Agilent 6530 QTOF MS (Agilent) was collected in negative mode, which was operated in full-scan mode at m/z 100 mm, 1.8 μm). The drying gas temperature was set at 350 °C. Capillary voltage and cone voltage were set at 3.5 kV and 20 V, respectively. The desolvation temperature was set at 350 °C. Nitrogen was used as both the cone gas (50 liters/h) and the desolvation gas (9 liters/min). Argon was used as collision gas. Collision energy ranging from 10 to 40 eV was applied for MS/MS fragmentation of BAs. Multivariate data analysis was performed by mass Profinder software (Agilent). Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were carried out using SIMCA-p + 13.0 (Umetrics, Kinnelon, NJ). HMDB and METLIN databases were used to determine the chemical structures of endogenous metabolites which were confirmed by comparing retention times and MS/MS fragmentation patterns with authentic standards (supplemental Fig. S1 and Table S1).

**RNA Analysis, Western Blot Analysis, and Immunoprecipitation**—QPCR and Western blotting analyses were carried as detailed in a previous report (21). Primer sets are shown in supplemental Table S2. Values were normalized to Actb mRNA or 18S ribosomal RNA. The expression of miR-34a-5p and U6sn RNA was also evaluated, and they were synthesized from Ribobio (Guangzhou, China). U6sn RNA was used as the normalization control for miRNAs. To determine acetylation status of FXR in liver homogenates, 500 μg liver extracts was incubated with FXR antibody (2 μg) overnight under stringent conditions, immunopurified using protein A/G plus-agarose (sc-2003, Santa Cruz Biotechnology), and immunoblotted using Ac-Lysine antibody. The following antibodies were used in Western blotting and immunoprecipitation: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14C10, Cell Signaling Technology, MA), SIRT1 (D1D7, Cell Signaling Technology), Normal mouse IgG (sc-2025, Santa Cruz Biotechnology), and anti-rabbit peroxidase-conjugated second antibody (SA00001–2, Proteintech, IL). RNA-Seq analysis was carried out using a method described previously (22).

**Biochemical Analysis and Histological Examination**—Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and adenosine triphosphate (ALP) in the mouse serum were measured by corresponding commercial kits in accordance with the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). H2O2 and malondialdehyde (MDA) in serum and liver were measured by commercial kits (Nanjing Jiancheng Bioengineering Institute). GSH levels in serum and liver were measured through LC-MS. Serum SIRT1 concentrations in clinical samples were determined using a commercially available human SIRT1 ELISA kit (Bio-Swamp, HM12168, Wuhan, China). Hematoxylin and eosin (H&E) staining and Sirius red staining were carried to assess liver injury and fibrosis development as detailed in a previous report (4).

**Preparation and Culture of Primary Mouse Hepatocytes**—Mouse primary hepatocytes were isolated from male C57BL/6J using two-step perfusion method and cultured as described (23). Cells were harvested after incubation with DCA (10, 50, 100, 200, 400 μM) (24), TCA (100 μM) (12), TDCA (100 μM), and celastrol (0.01, 0.1, 1.0 μM) (25) for 24 h. After 24 h incubation, cell viability in primary cells was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). The absorbance of dissolved formazan grains was measured at 570 nm.

**Data Analysis**—The data were shown as the mean ± S.E. and processed with GraphPad Prism (San Diego, CA). Statistical analysis was performed using the one-way ANOVA followed by Dunnett’s test (for multiple groups) or the two-tailed Student t test (for two groups). A p < 0.05 was considered statistically significant.

**RESULTS**

**Serum Metabolomic Analysis of Human and Mouse Cholestasis**—To discover metabolites that are changed in cholestasis, PCA and OPLS-DA models were used to analyze the serum metabolites from patients and healthy volunteers. Significant differences in a number of the metabolites between these two groups were found, with increased BAs largely contributing to the separation of patients and health volunteer groups (Fig. 1A). Levels of the four ions (m/z, 448.3068 at 8.64 min; m/z, 498.2895 at 8.39 min; m/z, 464.3017 at 7.59 min; m/z, 514.2843 at 7.20 min), identified as GCDCA, TDCA, GCA, and TCA, were significantly increased. Identification of the ions was performed by tandem mass spectrometry MS/MS fragmentation and column retention times compared with authentic standards (supplemental Fig. S1). Further analysis indicated that the levels of seven free and conjugated BAs, CA, DCA, TCA, GCA, TDCA, GDCA, and GCDCA were significantly increased in the serum of cholestatic patients (Fig. 1B and 1C). Screening standard for BA targeted metabolomics analysis can be found in supplemental Materials and Methods. Similarly, PCA analysis of serum samples revealed significant separation among the control, ANIT, and TAA groups (Fig. 1D). The contribution analysis of the loading plot again indicating BAs as the primary commonly increased ions contributing to the separation (Fig. 1D). Further targeted metabolomics analysis showed that seven BAs were significantly increased in both ANIT- and TAA-induced cholestasis (Fig. 1E).

**DCA Inhibits the SIRT1 Activity in Cholestasis**—Serum chemistry analysis indicated that the key clinical factors related to cholestasis, including ALP, γ-glutamyltransferase (GGT), BAs, and total bilirubin (TBIL) levels, were significantly higher in the cholestatic patients compared with healthy volunteers, indicating the existence of severe cholestasis in these patients (Table I). Additionally, albumin (ALB) and cholesterol (CHOL) levels were significantly decreased in the cholestatic patients (Table I). Serum SIRT1 activity was significantly inhibited in cholestatic disease patients compared with the healthy volunteers (Table I). In the mouse cholestatic
disease models, hepatic Sirt1 mRNA expression was decreased in both ANIT- and TAA-induced cholestatic liver injury (Fig. 2A).

Previous studies reported that some BAs can decrease SIRT1 protein levels in primary rat hepatocytes (24). DCA, TCA, and TDCA were three of the commonly increased BAs in human and mouse cholestasis (Fig. 1B, 1C, 1E). In vitro assays indicated that 100 μM DCA, TCA, and TDCA decreased Sirt1 mRNA levels, with DCA showing the maximal inhibition of Sirt1 mRNA (supplemental Fig. S2). DCA decreased Sirt1 mRNA in a dose-dependent manner from 100 to 400 μM (Fig. 2B). More importantly, the level of SIRT1 protein was de-
creased after treatment with 100 and 200 μM DCA (Fig. 2E). Celastrol, a pentacyclic triterpene found in the roots of the *Tripterygium Wilfordii* (thunder grove vine), was able to increase Sirt1 mRNA levels (25). In agreement with a previous study, Sirt1 mRNA levels and SIRT1 protein levels were significantly increased and Ac-p53 levels were significantly decreased by 0.1 and 1.0 μM celastrol in primary mouse hepatocytes (Fig. 2C, 2E). Further, 1.0 μM celastrol prevented the decrease of Sirt1 mRNA and SIRT1 protein by DCA (200 μM), suggesting that the inhibition of SIRT1 by DCA could be ameliorated by celastrol (Fig. 2D and 2E).

Finally, DCA induced the miR-34a/Sirt1/p53 proapoptotic pathway, and celastrol reversed the proapoptotic pathway (supplemental Fig. S3).

**Celastrol Protects Against ANIT-induced Cholestasis**—As SIRT1 activity was downregulated in ANIT-induced cholestasis, celastrol might protect the mice from cholestasis by increasing SIRT1 levels. As expected, hepatic histological analysis showed that celastrol alleviates inflammatory infiltration and parenchymal necrosis induced by ANIT (Fig. 3A). Consistent with the histology, the increased liver/body weight ratio and the weight loss induced by ANIT were attenuated by celastrol (supplemental Fig. S4B and S4C). Further, the increased AST, ALT, and ALP in ANIT-induced cholestasis were decreased by celastrol (Fig. 3B). Celastrol inhibited hepatic expression of mRNAs encoding inflammatory factors in ANIT-induced cholestasis, including interleukin-6 (Il6) and tumor necrosis factor α (Tnfα) (Fig. 3C). Further, celastrol could reduce ANIT-induced hepatocyte apoptosis, fibrosis, ER stress, and oxidative stress (supplemental Fig. S5).

Using ANIT exposure in mice as a model for cholestasis, targeted analysis of the levels of individual BAs in serum and liver was carried out (supplemental Fig. S4D and S4E). The increased levels of CA, DCA, TβMCA, TαMCA, TCA, THDCA, and TUDCA in serum of ANIT-treated mice were significantly attenuated by celastrol (supplemental Fig. S4D). The increased levels of ωMCA, βMCA, CA, DCA, TβMCA, TαMCA, TCA, and TUDCA in liver of ANIT-treated mice were recovered by celastrol (supplemental Fig. S4E). The expression of Sirt1, Fxr, and BA biosynthesis and transporter-encoding mRNAs were also evaluated. Celastrol treatment significantly increased Sirt1 and Fxr mRNA levels compared with the ANIT-treated group (supplemental Fig. S4F). The level of a BA synthesis gene mRNA, Cyp7a1, decreased in the ANIT-treated group was recovered by celastrol treatment (supplemental Fig. S4F). The expression of two basolateral uptake transporters, sodium taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide 4 (Oatp4) mRNAs were significantly decreased in the ANIT-treated group, whereas the levels of these mRNAs were increased by celastrol (supplemental Fig. S4F).

To further explore the mechanism associated with the protective effect of celastrol on ANIT-induced cholestasis, global gene expression differences in the liver of ANIT and ANIT+Celastrol groups were assessed by RNA-Seq, and a total of 978 differentially expressed genes (DEGs) were identified (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110391; GSE110391) (supplemental Fig. S6). Large numbers of these DEGs were related to activation of SIRT1, which included increased FXR signaling and inhibition of PPARγ, nuclear factor-kappa B (NF-κB), and P53 signaling (Fig. 3D). The protein levels of SIRT1 and its target gene Ac-FXR, FXR, NF-κB, Ac-p53, and p53 were also recovered by celastrol (Fig. 3F and 3G). In addition, Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that celastrol affected the metabolism of lipids, inflammation, and BA homeostasis (Fig. 3E). Among these pathways, the functions of bile secretion, primary BA biosynthesis, and ABC transporters were closely related to the homeostasis of BAs and the signaling pathway of FXR. These data demonstrate that celastrol may protect against ANIT-induced cholestasis, and its protective effects might be dependent on activation of SIRT1 and FXR.

**Celastrol Protects Against TAA-induced Cholestasis**—TAA exposure can induce cholestatic liver damage through the accumulation of BA, which may cause the liver fibrosis leading to cirrhosis. Here, the effect of celastrol on TAA-induced cholestasis was tested. H&E staining of liver sections revealed that celastrol treatment attenuated TAA-induced necrotic foci and congestion (supplemental Fig. S7A and S7B). Consistent with these results, serum AST, ALT, and ALP levels in the TAA+Celastrol group were decreased compared with the TAA group (supplemental Fig. S7C). Additionally, celastrol reduced the elevated levels of inflammatory factors by TAA exposure, such as Il1 and Il6 mRNAs (supplemental Fig. S7D).

The increased levels of ωMCA, DCA, TβMCA, TαMCA, TCA, THDCA, TUDCA, TCDDA, and TDCDA in serum of TAA-treated mice were significantly attenuated by celastrol (Fig. 4A). The increased levels of ωMCA, βMCA, CA, DCA, TβMCA, TαMCA,
TCA, and TLCA in livers of TAA-treated mice were recovered by celastrol (Fig. 4B). The mRNA levels of Sirt1 and Fxr decreased in the TAA-treated group were recovered by celastrol (Fig. 4C). The levels of Cyp7a1, Cyp8b1, Ntcp, multidrug resistance protein 4 (Mrp4), bile salt export pump (Bsep), and Mrp2 mRNAs decreased in the TAA group, were increased by celastrol (Fig. 4C). The protein levels of SIRT1 and its target gene Ac-FXR, FXR, NF-κB, Ac-p53, and p53 were also recovered by celastrol (Fig. 4D and 4E). In conclusion, these results demonstrate that celastrol could prevent TAA-induced cholestatic liver injury.

Role of SIRT1 in Celastrol Protection Against Cholestasis—To determine whether celastrol protected against cholestasis through the activation of SIRT1, a selective SIRT1 inhibitor EX527 was used to decrease SIRT1 activity in the mouse. EX527 could decrease SIRT1 protein levels and increase Ac-p53 protein levels in mice, indicating the efficacy of EX527 (supplemental Fig. S9A). PCA analysis showed that the ANIT+Celastrol+EX527 group deviated from the control and ANIT+Celastrol groups, indicating that EX527 diminished the protective effects of celastrol (supplemental Fig. S8C). H&E staining results demonstrated that co-treatment with EX527 markedly increased the ANIT-induced necrotic foci in the liver compared with the ANIT+Celastrol group (Fig. 5A and supplemental Fig. S8A). Fibrosis was not obvious in the ANIT model (Fig. 5B). Consistent with the H&E staining results, serum AST and ALT levels in the ANIT+Celastrol+EX527 group were increased compared with the ANIT+Celastrol...
Activation of SIRT1-FXR Improves Cholestasis

A

Control

40x

100x

ANIT

40x

ANIT+Celastrol

40x

100x

B

Enzyme activity (U/L)

Control

ANT

ANT+Celastrol

 AST

ALT

ALP

C

Relative expression of mRNA (Fold)

Control

ANT

ANT+Celastrol

II1

II6

Tnfα

D

E

Chemical carcinogenesis
Retinol metabolism
Metabolism of xenobiotics by cytochrome P450
Bile secretion
Complement and coagulation cascades
Cell adhesion molecules (CAMs)
Phagosome
Drug metabolism - other enzymes
Ascorbate and aldarate metabolism
Linoleic acid metabolism
Steroid biosynthesis
Biosynthesis of unsaturated fatty acids
Pentose and glucuronate interconversions
Fluid shear stress and atherothrombosis
Arachidonic acid metabolism
Fatty acid elongation
MicroRNAs in cancer
NOD-like receptor signaling pathway
PPAR signaling pathway
Primary bile acid biosynthesis
ABC transporters
Osteoclast differentiation
Inflammatory bowel disease (IBD)

F

G

NF-κB p65
Ac-p53
p53
Sirt1
GAPDH

56 kDa

Control

ANIT

ANIT+Celastrol

Input: Fxr

IP: IgG

IP: Fxr, IB: Ac-Lys

Control

ANT

ANT+Cela

Control

ANT

ANT+Cela

Relative amount (Sirt1/GAPDH)

Control

ANT

ANT+Cela

Control

ANT

ANT+Cela
Further, co-treatment with EX527 increased the levels of various serum BAs compared with the ANIT/H11001 Celastrol group (Fig. 5D). Cotreatment with EX527 also decreased the expression of \textit{Fxr}, \textit{Ntcp}, and \textit{Oatp4} mRNAs, decreased the expression of SIRT1 protein levels, increased the expression of Ac-p53 protein levels compared with the ANIT/H11001 Celastrol group (supplemental Fig. S8D and S9B). Additionally, EX527 also diminished the protective effects of celastrol on TAA-induced cholestasis, as indicated by serum metabolomics analysis, liver histology, increased AST and ALT, and ALP enzyme activity.

**Fig. 4.** Celastrol attenuated TAA-induced cholestasis in WT mice. Celastrol decreased the accumulation of BAs in serum (A) and liver (B). C, QPCR analysis of the hepatic Sirt1, Fxr, Cyp7a1, Cyp8b1, Ntcp, Oatp4, Mrp4, Bsep, and Mrp2 mRNAs. All data were plotted as mean ± S.E. (n = 6). **p < 0.01 and ***p < 0.001 versus control; "p < 0.05, **p < 0.01, and ***p < 0.001 versus TAA. D, Western blotting was used to measure FXR, NF-κB, Ac-p53, p53, SIRT1 levels. **p < 0.01 and ***p < 0.001. E, Acetyl-FXR levels were significantly elevated in TAA group and significantly decreased in TAA+Celastrol group as assessed using immunoprecipitation.

**Fig. 3.** Celastrol attenuated ANIT-induced cholestasis in WT mice. A, H&E staining of liver. Solid arrow: focal necrosis. B, Serum AST, ALT, and ALP enzyme activity. C, QPCR analysis of inflammatory factors in liver. All data were repressed as mean ± S.E. (n = 6). ****p < 0.001 versus control; "p < 0.05, **p < 0.01, and ***p < 0.001 versus ANIT. D, Heat map display of 27 gene (mRNAs) obtained by RNA-Seq that correlated with the SIRT1 pathway. E, Pathway enrichment analysis of the DEGs. F, Western blotting measurement of FXR, NF-κB, Ac-p53, p53, SIRT1 levels. **p < 0.01. G, Acetyl-FXR levels were significantly elevated in ANIT group and significantly decreased in ANIT+Celastrol group as assessed using immunoprecipitation.
Fig. 5. EX527 reduced the protective effect of celastrol in both ANIT- and TAA-induced cholestasis in WT mice. A, H&E staining of liver in ANIT- and TAA-induced cholestasis. Solid arrow: focal necrosis. B, Sirius red staining of liver in ANIT- and TAA-induced cholestasis. C, Serum AST, ALT, and ALP enzyme activities in ANIT-induced cholestasis. D, Serum BA levels in ANIT-induced cholestasis. E, Serum AST, ALT, and ALP enzyme activities in TAA-induced cholestasis. F, Serum BAs levels in TAA-induced cholestasis. All data were repressed as mean ± S.E. (n = 4). *p < 0.05, **p < 0.01, and ***p < 0.001 versus control; δp < 0.05, δδp < 0.01, and δδδp < 0.001 versus ANIT/TAA; δδδδp < 0.001 versus ANIT/Celastrol/TAA/Celastrol.
ALT, and BA levels, decreased Fxr, Cyp8b1, and Ntcp mRNAs, decreased SIRT1 protein levels, and increased Ac-pS3 protein levels compared with the TAA+Celastrol group (Fig. 5, supplemental Fig. S8 and S9).

Another SIRT1 inhibitor salermide was also used. Salermide diminished the protective effects of celastrol on ANIT- and TAA-induced cholestasis, as indicated by increased AST and ALP, and BAs levels (Fig. 6). Additionally, the SIRT1 agonist SRT1720 was found to reduce ANIT-induced cholestasis in previous study (25). Celastrol showed similar therapeutic effects as SRT1720 in TAA-induced cholestasis (Fig. 6). Co-treatment with SRT1720 and celastrol significantly enhanced the protective effect of SPT1720 (Fig. 6). These results suggested that the protection of celastrol against cholestasis was dependent on SIRT1 activation.

**FXR Disruption Attenuates the Protection of Celastrol Against Cholestasis**—Because SIRT1 is a critical regulator of FXR that can be affected by cholestasis, a role for FXR in protection against cholestatic liver damage was examined. Fxr-null mice were administered with ANIT and celastrol. Serum metabolomics analysis showed that the ANIT+Celastrol group was similar to the control group in WT mice, whereas the ANIT+Celastrol group in Fxr-null mice was similar to the ANIT group (Fig. 7A and 7B). H&E staining revealed that the liver histology was not attenuated by celastrol in Fxr-null mice (supplemental Fig. S10). The decreased ALT, ALP, and BA levels, decreased Fxr, Cyp8b1, and Ntcp mRNAs, decreased SIRT1 protein levels, and increased Ac-pS3 protein levels compared with the TAA+Celastrol group (Fig. 5, supplemental Fig. S8 and S9).

![Graphs showing enzyme activities and bile acids levels](image_url)
levels with celastrol treatment in WT mice were also not observed in the Fxr-null mice (Fig. 7C and 7D). The increase of Cyp7a1 and Oatp4 mRNAs in WT mice were abrogated in Fxr-null mice (supplemental Fig. S10B). Additionally, FXR disruption also diminished the protective effect of celastrol in TAA-induced cholestasis, as indicated by serum metabolomics analysis, liver histology, AST, ALP, and BA levels, and Cyp7a1, Cyp8b1, Ntcp, Bsep, and Mrp2 mRNA levels in Fxr-null mice (Fig. 7 and supplemental Fig. S11). These results suggested that the SIRT1 target gene FXR may be responsi-
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The present study demonstrated a protective role for SIRT1 during cholestatic liver damage, and sheds new insights into the significance of the SIRT1 and FXR signaling in mediating these protective effects. SIRT1 activity was inhibited in both human and mouse cholestasis. Mass spectrometry-based metabolomics was engaged to examine the changes of endogenous metabolites in serum during cholestasis and revealed that an increase in the toxic BAs (DCA, TCA, and TDCA) during cholestasis were potentially responsible for the decreases in SIRT1 activity. Interestingly, the decrease of SIRT1 activity by DCA was inhibited by celastrol in the experimental mouse cholestasis model and in in vitro assays, leading to attenuation of cholestatic liver damage. Further studies in Fxr-null mice and EX527- and salermide-treated WT mice revealed that the protective role of celastrol was dependent on activation of the SIRT1-FXR signaling pathway (Fig. 7).

Celastrol is a triterpene compound obtained from the roots of the *Tripterygium Wilfordii* (thunder god vine) (17). Celastrol represented a promising treatment option for mesangioproliferative glomerulonephritis and other glomerular diseases (26). Celastrol improved obesity and metabolic dysfunction via the activation of heat shock factor 1-peroxisome proliferator-activated γ coactivator-1α axis in mice (27). The effects of celastrol on SIRT1 agree with an earlier study which demonstrated that celastrol protected against high-fat-diet induced fatty liver via promoting the expression of SIRT1 (25). The present study indicates that the SIRT1 activity was decreased in both human and mouse cholestasis, consistent with a recent clinical observation (28). Thus, the question arose as to whether celastrol can protect against cholestatic liver damage through the activation of SIRT1. Here, metabolomics revealed that a series of BAs were elevated in the serum and liver of human cholestasis and experimental mouse models. Interestingly, DCA was found to be an inhibitor of SIRT1 in this work and an earlier study (24). The present study revealed that Sirt1 mRNA and SIRT1 protein levels were significantly inhibited in the ANIT- and TAA-treated groups as a result of increased DCA. Celastrol not only inhibited the decrease of SIRT1 by DCA in hepatocytes, but also elevated the level of SIRT1 in mouse liver cholestasis that was decreased by ANIT and TAA treatment. In agreement with an earlier study, SIRT1 activation may alleviate CA diet-induced cholestasis (29). Celastrol treatment could decrease the accumulation of BAs and hepatic inflammation in ANIT- and TAA-induced cholestasis. When co-administered with the SIRT1 inhibitor EX527 and salermide, the protective effects of celastrol on cholestasis were diminished, suggesting that its protective role is dependent on SIRT1. Thus, the question arose how celastrol increases SIRT1. In fact, other studies demonstrated that celastrol shows dramatic protective effects on obesity, fatty liver, and metabolic dysfunction-induced by high-fat-diet in mice (17, 25, 27). It was reported that celastrol modulates several cell signaling pathways, including the MAPK pathway, PI3K/Akt/mTOR pathway, JAK/STAT pathway, NF-κB pathway, and antioxidant defense mechanisms (30). Among these signaling pathways, MAPK is frequently involved in the effects of SIRT1, including neuroprotective role, astrocyte activation, inhibition of apoptosis and anti-oxidative stress (31–33). It is likely that the combined activities of SIRT1 and MAPK contribute to the protection of celastrol, and their roles specifically at the level of maintaining BA homeostasis warrant further investigation.

One of the intriguing findings in this study was the significant decrease in SIRT1 in human and mice cholestasis. More importantly, the present study demonstrates that the increase in DCA is responsible for the inhibition of SIRT1, and its recovery by celastrol results in alleviation of cholestasis. SIRT1 is an important silent information regulator and an NAD-dependent protein deacetylase, which affects numerous processes such as inflammation, metabolism, and growth (29). It was reported that SIRT1 may regulate NF-κB and several nuclear receptors, including FXR, PPARα, and PPARγ (29, 34). Although other studies reported a protective role for SIRT1 in aging, limited studies investigated the correlation between SIRT1 and cholestasis. SIRT1 activates many target genes primarily associated with deacetylation (35, 36). Thus, it was important to determine which among these target genes afforded protection against cholestasis. Earlier studies revealed that SIRT1 was a critical transcriptional and trans-activational regulator of FXR (37). It modulates FXR activity by deacetylating the protein and histones. Notably, FXR Lys217 was the major deacetylation site regulated by SIRT1 (37). In addition to direct regulation of FXR, SIRT1 could indirectly regulate the deacetylation of FXR through hepatocyte nuclear factor 1α (HNF1α), which can bind to the Fxr promoter. Hepatic and intestinal SIRT1 deficiency decreased the expression of FXR along with its target genes largely through the downregulation of HNF1α (38, 39). FXR is known to be the master sensor of intracellular levels of BAs in liver and intestinal epithelial cells and a crucial control element for maintaining BA homeostasis and is a recognized therapeutic target for cholestasis (40). In the present study, whole body FXR deficiency attenuated the protective effects of celastrol on cholestasis, as indicated by liver histology, BA levels, and its relevant gene expression patterns. The fact is that there are many complications with obesity, including increased fatty liver, gallstones, hyperlipidemia, and insulin resistance (41). SIRT1 activation improves the metabolic syndrome (42, 43). Recent studies reported that SIRT1 expression was downregulated in cholestasis patients (28) and obese patients with nonalcoholic fatty liver disease (44, 45). Therefore, SIRT1-FXR signaling could be a promising therapeutic target for the cholestasis, especially for the obese patients with cholestasis.
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Considering that Fxr-null mice had some unusual phenotypes, including disrupted multiple neurotransmitter systems and neurobehavior (46), impaired urine concentrating ability (47), and changed gut microbiota (48), FXR may affect various pathways in vivo. Liver- and intestine-specific Fxr-null mice were developed to show differential roles of this receptor (49–51). A previous study found that intestine-specific Fxr-null mice were resistant to obesity and insulin resistance compared with wild-type mice, suggesting that the intestinal FXR mediates the anti-obesity effects (51). Intestine-specific Fxr-null mice also could reduce hepatic triglyceride accumulation in response to a high-fat diet, indicating that intestinal FXR is a potential therapeutic target for nonalcoholic fatty liver disease (50). Further, using liver- and intestine-specific Fxr-null mice, it was found their differential regulation of bile acid homeostasis (49). Activation of FXR in intestine but not liver was required for short-term repression of CYP7A1 in liver, and CYP8B1 was more dependent on the presence of FXR in liver (49). Therefore, hepatic-specific Fxr-null mice would be necessary to determine the role of hepatic FXR in the therapeutic effect of cerastrol in the future.

In conclusion, metabolomics revealed that inhibition of SIRT1 in human and mouse cholestasis was associated with the accumulation of DCA, TCA, and TDCA. Using Fxr-null mice and SIRT1 inhibitor intervention, a convincing role for the accumulation of DCA, TCA, and TDCA. Using Fxr-null mice and SIRT1 inhibitor intervention, a convincing role for the accumulation of DCA, TCA, and TDCA. Using Fxr-null mice and SIRT1 inhibitor intervention, a convincing role for the accumulation of DCA, TCA, and TDCA. Using Fxr-null mice and SIRT1 inhibitor intervention, a convincing role for the accumulation of DCA, TCA, and TDCA. Therefore, hepatic-specific Fxr-null mice would be necessary to determine the role of hepatic FXR in the therapeutic effect of cerastrol in the future.

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DATA AVAILABILITY

RNA-Seq can be found in https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110391.

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