




Inverse agonist of ERR γ reduces cannabinoid receptor type 1-mediated induction of fibrinogen synthesis in mice with a high-fat diet-intoxicated liver

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Abstract

Upon liver intoxication with malnutrition or high-fat diet feeding, fibrinogen is synthesized by hepatocytes and secreted into the blood in human and mouse. Its primary function is to occlude blood vessels upon damage and thereby stop excessive bleeding. High fibrinogen levels may contribute to the development of pathological thrombosis, which is one mechanism linking fatty liver disease with cardiovascular disease. Our previous results present ERR γ as key regulator of hepatocytic fibrinogen gene expression in human. In a therapeutic approach, we now tested ERR γ inverse agonist GSK5182 as regulator of fibrinogen levels in mouse hyperfibrinogenemia caused by diet-induced obesity and in mouse hepatocytes. ACEA, a CB1R agonist, up-regulated transcription of mouse fibrinogen via induction of ERR γ , whereas knockdown of ERR γ attenuated the effect of ACEA (10 μ M) on fibrinogen expression in AML12 mouse hepatocytes. Deletion analyses of the mouse fibrinogen γ (FGG) gene promoter and ChIP assays revealed binding sites for ERR γ on the mouse FGG promoter. ACEA or adenovirus ERR γ injection induced FGA, FGB and FGG mRNA and protein expression in mouse liver, while ERR γ knockdown with Ad-shERR γ attenuated ACEA-mediated induction of fibrinogen gene expression. Moreover, mice maintained on a high-fat diet (HFD) expressed higher levels of fibrinogen, whereas cannabinoid receptor type 1 (CB1R)-KO mice fed an HFD had nearly normal fibrinogen levels. Finally, GSK5182 (40 mg/kg) strongly inhibits the ACEA (10 mg/kg) or HFD-mediated induction of fibrinogen level in mice. Taken together, targeting ERR γ with its inverse agonist GSK5182 represents a promising therapeutic strategy for ameliorating hyperfibrinogenemia.

Keywords Cannabinoid receptor · ERR γ · Fibrinogen · High-fat diet · Hyperfibrinogenemia · GSK5182

Introduction

The orphan estrogen-related receptors (ERRs) are members of the NR3B subfamily of nuclear receptors, which consists of three members: ERR α , ERR β and ERR γ . The

ERRs are closely related to estrogen receptors and are primarily expressed in brain, heart, kidney, liver, pancreas, placenta and skeletal muscle (Hong et al. 1999; Lui et al. 2006; Misra et al. 2017; Zhang and Teng 2007). Recent studies have reported that hepatic ERR γ regulates hepatic

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gluconeogenesis and hepatic insulin signaling, leading to type 2 diabetes mellitus (T2DM), alcohol-induced oxidative stress and liver injury (Kim et al. 2011, 2012). Structural studies suggest that the ERRs are constitutively active in the absence of natural ligands, such that small molecule ligands could either further activate or repress ERR γ activity. ERR γ has attracted significant attention from the scientific community because of its potential roles in cancers and metabolic disease, and regarding discovery of novel ligands. 4-Hydroxytamoxifen (4-OHT) and diethylstilbestrol (DES), known as antagonist and agonist of ER α , were reported as inverse agonists of ERR β and ERR γ . This was confirmed by biochemical and X-ray crystallographic studies (Brzozowski et al. 1997). In addition, phenolic acyl hydrazones were reported as functional ERR γ agonists, although in-depth studies of its molecular basis and the observed agonistic response are still lacking (Zuercher et al. 2005). GSK5182 (4-[(Z)-1-[4-(2-dimethylaminoethoxy)phenyl]-hydroxy-2-phenylpent-1-enyl]phenol) is a specific inverse agonist for ERR γ , a member of the orphan nuclear receptor family that has important functions in development and metabolic homeostasis (Chao et al. 2006). GSK5182 has been shown to restore impaired insulin signaling by inhibiting diacylglycerol production. In addition, GSK5182 lowers blood glucose levels through the inhibition of hepatic gluconeogenesis and ameliorates hyperglycemia in diabetic mice by inhibiting ERR γ transcriptional activity. However, more therapeutic effects of GSK5182 are waiting to be explored in liver.

Fibrinogen (FG) is a plasma glycoprotein synthesized in the liver that is essential for haemostasis, wound healing, fibrinolysis, inflammation, angiogenesis, cellular and matrix interactions, and neoplasia. Elevated plasma fibrinogen is associated with risk of cardiovascular disease and arterial and venous thrombosis (Acevedo et al. 2002; Fibrinogen Studies et al. 2005; Kamphuisen et al. 1999; Kannel et al. 1987; Lindahl et al. 2000; Toss et al. 1997; van Hylckama Vlieg and Rosendaal 2003; Wilhelmssen et al. 1984; Yarnell et al. 1991). Several studies have detected dose effects, with increased risk of death or thrombosis in subjects with the highest plasma fibrinogen concentrations (Kamphuisen et al. 1999; Kannel et al. 1987; Toss et al. 1997; Yarnell et al. 1991). HNF-1 is a basal transcription factor for FGA and FGB expression in the rat, binding to recognizable consensus-like sequences in the proximal promoters of each (Courtois et al. 1987). Fibrinogen expression can be regulated in various physiological and pathophysiological situations. Synergistic regulation of acute phase response (APR) proteins by glucocorticoids and IL-6 has been widely described and includes regulation of fibrinogen expression (Otto et al. 1987). More recently, a circadian rhythm to fibrinogen levels was reported in asymptomatic human subjects (Bremner et al. 2000) and also in a study of mouse liver RNA and circulating fibrinogen antigen (Sakao et al.

2003). Moreover, a miRNA library was screened for activity in controlling expression of the fibrinogen genes (Fort et al. 2010). Therefore, fibrinogen is a potential diagnostic and therapeutic target for predicting and reducing thrombosis.

Endocannabinoids are lipid ligands that bind to cannabinoid receptors. The best characterized of these receptors are the cannabinoid 1 receptor and the cannabinoid 2 receptor (Cota et al. 2003). Although traditionally associated with the central nervous system (CNS), cannabinoid receptors in hepatocytes are increasingly being recognized as key mediators of fatty liver (Purohit et al. 2010) and associated insulin resistance (Chanda et al. 2011, 2012; Gary-Boho et al. 2007) caused by high-fat diet (Osei-Hyiaman et al. 2008a), viral hepatitis (Enjoji et al. 2012; Toyoda et al. 2011; van Hylckama Vlieg and Rosendaal 2003) and ethanol intake (Jeong et al. 2008). Additionally, patients with non-alcoholic fatty liver disease were found to have a huge increase in the amount of hepatic CB1R mRNA compared with patients without liver pathology (Liu et al. 2012). Liver-specific knockout of the CB1R made mice resistant to high-fat diet (HFD)-induced steatosis, although overall adiposity and weight gain were not affected in this situation (Osei-Hyiaman et al. 2008a). However, knockout of genes that control important metabolic regulators may lead to compensatory antenatal changes affecting appetite; thus, the knockout does not always have the same effects as inhibition of the corresponding metabolic regulators in adults.

In this study, we further confirmed the nuclear receptor ERR γ as a transcriptional regulator of hepatic fibrinogen gene expression *in vivo*. Here, activation of hepatic CB1R signaling induced ERR γ -mediated transcription of the fibrinogen gene in mice. Fibrinogen and ERR γ were more elevated in HFD mice than in normal chow diet (NCD) mice, and they were significantly lower in CB1R-KO mice fed an HFD. GSK5182 decreased HFD or ACEA (a selective CB1R agonist)-mediated induction of fibrinogen level in mice. Based on these findings, inhibition of the transcriptional activity of ERR γ by GSK5182 may have the potential to ameliorate hyperfibrinogenemia.

Materials and methods

Animal studies

CB1R-KO mice, described previously (Kim et al. 2013), were kindly provided by Dr. George Kunos at the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Male 8-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used for experiments. The mice were acclimatized to a 12-h light/dark cycle at 22 ± 2 °C with free access to food and water. Ad-GFP and Ad-FLAG-ERR γ were injected into the tail veins of mice, which were killed

on day 3 after injection. To determine the effect of ERR γ , control and recombinant shERR γ adenoviruses were injected into mice in the presence or absence of ACEA (10 mg/kg intraperitoneal injection). Mice were killed at day 5 after adenovirus injection. For the GSK5182 study, mice were fed with normal chow diet (NCD) or a HFD; Research Diet D12492I, 60% kcal from fat for 15 weeks. GSK5182 (40 mg/kg, once daily) was administered to mice by i.p. injection for the last 2 weeks before killing. For CB1R-KO studies, 8-week-old WT and CB1R-KO mice were fed a NCD or HFD. Animals were weighed and food intake was measured. After 16 weeks of feeding, animals were killed, and liver tissue and blood were collected for further analysis. All animal experiments involved in this study were reviewed and approved by the Institutional Animal Use and Care Committee of Chonnam National University.

Chemicals and plasmids

GSK5182 was synthesized as previously described with slight modifications (Chao et al. 2006). ACEA was purchased from Tocris Bioscience. The promoters of mouse *FGG* (– 2 kb/+ 238 bp, – 1.3 kb/+ 238 bp, – 1 kb/+ 238 bp and – 0.6 kb/+ 238 bp) were cloned into the *XhoI/MluI* sites of the PGL3-basic vector. These reporter plasmids were confirmed by DNA sequencing. Expression vector for FLAG-ERR γ was described previously (Kim et al. 2012). A mutation was introduced into the ERRE of the mouse *FGG* promoter by site-directed mutagenesis (Stratagene, La Jolla, CA, USA), and the resultant construct (MT ERRE1-luc, MT ERRE2-luc and MT ERRE1&2-luc) was confirmed by DNA sequencing.

Recombinant adenovirus

Ad-GFP, Ad-FLAG-ERR γ , Ad-USi, and Ad-shERR γ were described previously (Kim et al. 2013). All viruses were purified using CsCl₂ or an Adeno-X maxi purification kit (Clontech, Palo Alto, CA, USA). For adenoviral infections, cells were washed with PBS and left for 2–3 h in serum-free medium containing the appropriate number of viral particles (multiplicity of infection = 100). The medium was replaced with fresh growth medium and the cells were grown for an additional 36–72 h before treatment.

Cell culture and transient transfection assay

AML12 cells (immortalized mouse hepatocyte) were maintained as described previously (Kim et al. 2011). The cells were used for experiments at 80% confluence. Transient transfections were conducted as described previously (Kim et al. 2011). Luciferase activity was normalized to β -galactosidase activity.

Measurement of fibrinogen level

Fibrinogen was extracted from cell culture medium and mouse blood, and its levels were determined using the Fibrinogen SimpleStep ELISA Kit (Abcam, Cambridge, MA, USA). Three replicate wells are used for standards and samples. The intra-assay ELISA coefficient of variation (CV) was 8.7%.

Isolation and culture of primary mouse hepatocytes

Mouse primary hepatocytes were isolated from the livers of 7-week-old male C57BL6 mice. The mice were anesthetized with Zoletile and their livers were exposed surgically. The liver was first perfused with resuspension buffer and then perfused with collagenase solution. Subsequently, the liver was finely chopped in a Petri dish and then filtered through 85- μ m pore mesh. Hepatocytes were collected by centrifugation at 800g for 2–5 min at 4 °C. Hepatocyte viability was assessed by trypan blue exclusion assay and was consistently in excess of 85%. Hepatocytes were then seeded onto collagen type 1-coated 60-mm dishes.

ChIP assay

The ChIP assay was performed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). Immunoprecipitation was performed using ERR γ antibody or IgG (as a negative control). After recovering the DNA, semi-quantitative PCR was performed using primers encompassing the mouse *FGG* promoter region. The primers used for polymerase chain reaction (PCR) were as follows: (– 1.7 kb/– 1.9 kb) forward 5'-CTGAGGCTTTGAGAGTGA TT-3' and reverse 5'-CCACAGCCAAGTAAGTAAAG-3' (– 0.8 kb/– 1.0 kb); forward 5'-CTGATGTTTTCTTTCTC CT-3' and reverse 5'-GGAGGATAGAAGATGGAGTT-3'.

Real-time PCR

Total RNA from hepatocytes or liver tissues was extracted using an RNA extraction kit. cDNAs were generated with the Maxime RT PreMix Kit (Intron Biotech) and qPCR using a SYBR Green PCR kit and AB Real-Time System (Applied Biosystems, Foster City, CA, USA). All data were normalized to actin expression.

Western blot analysis

Western blot analyses were performed as described previously (Kim et al. 2011). Cell lysates were prepared from hepatocytes or liver tissues of experimental animals, and Western blotting was performed using the indicated antibodies. The following primary antibodies were used for

the immunoblotting assay: beta-actin (AbFrontier, LF-PA0207A, Seoul, Korea), anti-FLAG M2 (Sigma Chemical Co., F3165, MO, USA), anti-ERR γ (Perseus Proteomics, PP-H6812-00, Tokyo, Japan) and Fibrinogen (Dako, A0080, Denmark). Using secondary antibodies conjugated with HRP, chemiluminescent western blot signals were captured with X-ray film.

Histological analysis

Livers were excised from mice fed NCD or HFD. For oil red O staining, liver tissues were embedded in a Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) and sectioned at a thickness of 8 μ m using a cryotome (Sakura Finetek). Cryostat sections of liver tissue were fixed in 10% neutral buffered formalin at room temperature. After fixation, liver tissue sections were stained with 0.3% oil red O solution for 1 h and counterstained with Mayer's hematoxylin for 30 s at room temperature. For hematoxylin and eosin (H&E) staining, liver samples were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5- μ m-thick sections, and stained with H&E. To detect fibrinogen or ERR γ , after blocking with CAS-Block (Invitrogen, Carlsbad, CA, USA), paraffin-embedded liver sections were incubated overnight at 4 $^{\circ}$ C with anti-fibrinogen (ab27913; Abcam, Cambridge, MA, USA) and anti-human ERR γ antibodies (Rabbit anti-ERR γ serum) were generated using a peptide (404-AGQHMEDPRRAGKMLM-419) from mouse ERR γ helix 9 (AbFrontier/Young in Frontier, Seoul, Korea), and visualized using 3,3'-diaminobenzidine. All images were captured using a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

Statistics

The data met assumptions of a normal distribution as determined by statistical software, and variance was estimated with SEM. The unpaired Student's *t* test was used for statistical analysis between two groups, whereas statistical significance between multiple-treatment groups was determined by analysis of one-way ANOVA. Mann–Whitney *U* or Kruskal–Wallis nonparametric analysis was used for *in vivo* studies. Differences were considered statistically significant at $p < 0.05$.

Results

Fibrinogen mRNA levels are increased by ACEA in mouse liver

Our previous study has delineated the CB1R–ERR γ –fibrinogen pathway in human hepatocytes. To confirm that this is

a general cross-species mechanism, we here tested mouse hepatocytes and mouse liver. ACEA increases fibrinogen mRNA in AML12 cells, starting at 3 h, with a peak at 24 h (Fig. 1a). Consistent with mRNA data, fibrinogen and ERR γ protein levels are increased upon 24-h ACEA treatment (Fig. 1b). Moreover, elevated fibrinogen levels are present in cell culture medium upon 24-h ACEA treatment (Fig. 1c). Finally, fibrinogen mRNA levels are induced in ACEA-injected C57BL/6J mice, as measured by real-time PCR for FGA, FGB and FGG mRNA. FGA mRNA levels reach a peak at day 5, while FGB and FGG reach maximum at day 3 (Fig. 1d). Taken together, these results demonstrate that the CB1R-mediated induction of fibrinogen expression is conserved in mice.

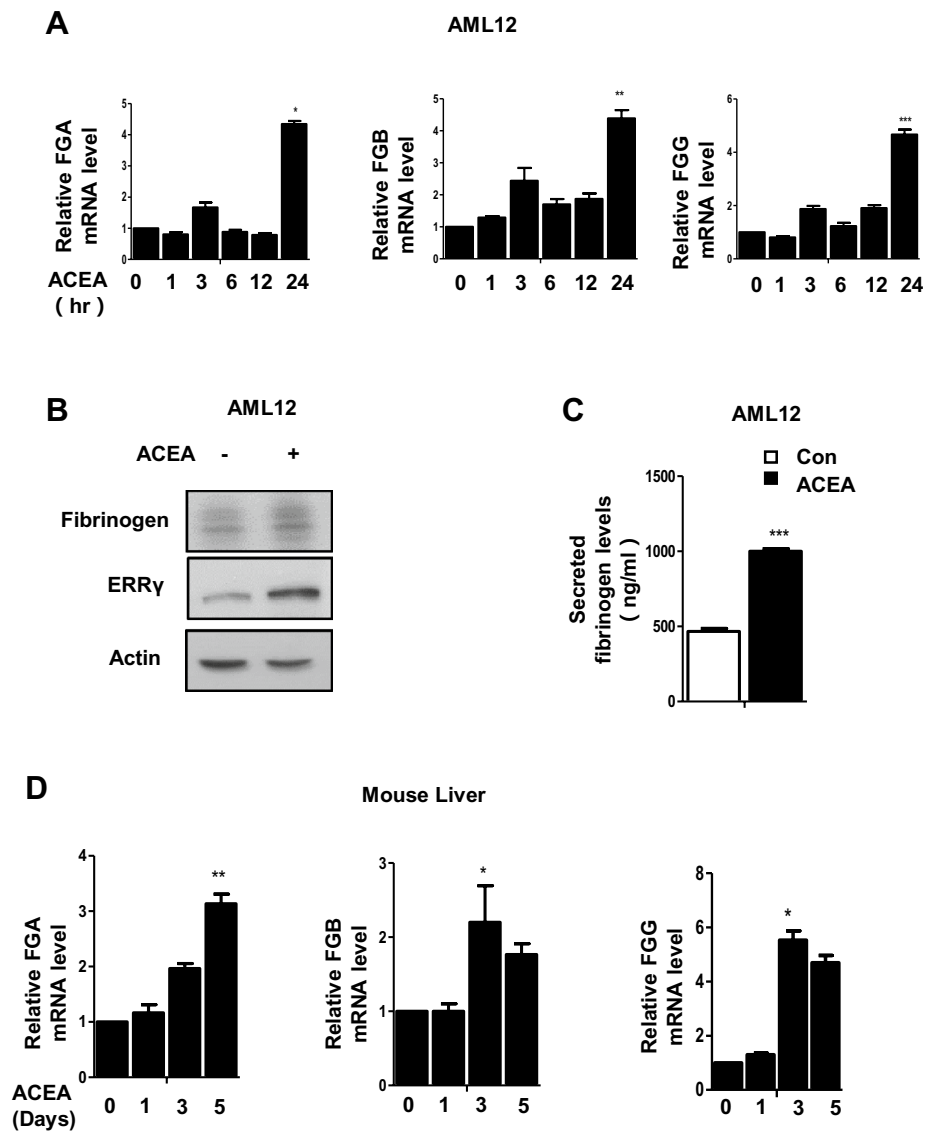
ERR γ induces fibrinogen expression in mouse liver

We examined whether ERR γ regulates fibrinogen expression in mouse primary hepatocytes. Adenoviral overexpression of ERR γ markedly increased fibrinogen protein levels in mouse primary hepatocytes (Fig. 2a). To verify the effect of ERR γ on fibrinogen secretion, we measured fibrinogen levels in cell culture medium from mouse primary hepatocytes infected with Ad-GFP and Ad-ERR γ . Ad-ERR γ significantly increased fibrinogen levels in the culture medium (Fig. 2b). Next, we examined the fibrinogen gene expression in liver after adenoviral overexpression of ERR γ . Ad-ERR γ -infection in mice increases hepatic fibrinogen mRNA and protein expression (Fig. 2c, d). These results suggested that ERR γ regulates fibrinogen expression in mouse liver.

Activation of CB1R induces fibrinogen gene expression via ERR γ in mice

To link ERR γ and the CB1 receptor for hepatic fibrinogen gene expression, we examined ACEA-induced fibrinogen expression upon knocking down of ERR γ using an adenovirus harboring ERR γ -targeting shRNA (Ad-shERR γ) in AML12 cells and in mice. The ACEA-induced fibrinogen mRNA level was dramatically reduced after depleting ERR γ in AML12 cells (Fig. 3a). Furthermore, ACEA-induced fibrinogen secretion in culture media of AML12 cells was significantly decreased by shERR γ (Fig. 3b). Then, mice were infected with Ad-shERR γ or Ad-Usi and treated with/without ACEA. ACEA-induced fibrinogen mRNA levels were significantly reduced in Ad-shERR γ -infected mouse liver (Fig. 3c). Moreover, the ACEA-induced fibrinogen protein level in mouse liver was also significantly reduced by knockdown of ERR γ (Fig. 3d). These results suggested that ERR γ is a key regulator of CB1R-mediated fibrinogen gene expression in mice.

Fig. 1 Activation of CB1R induces mouse fibrinogen gene expression in mouse hepatocyte and mice. **a–c** AML12 cells were treated with ACEA (10 μ M) for the indicated period. Total mRNA and proteins were isolated and used for qPCR and western blots. Cell culture medium was collected for the measurement of fibrinogen levels * p <0.05, ** p <0.01, *** p <0.001 by Student's *t* test. **d** ACEA administration (10 mg/kg, intraperitoneal injection) was performed in wild-type mice (n =5 per group) with indicated time. qPCR analysis was performed to measure mRNA levels in liver. * p <0.05, ** p <0.01 by Mann–Whitney *U* and Kruskal–Wallis tests



ERRγ activates the mouse fibrinogen γ gene promoter

To confirm a direct stimulating effect of ERR γ in ACEA-mediated induction of fibrinogen expression, we tested serial deletion constructs of the mouse fibrinogen γ promoter in reporter assays. The region spanning -1.0 to -0.6 kb is activated by ERR γ (Fig. 4a). Two putative ERR γ -binding motifs are present in the m-FGG promoter (AGGTGA as ERRE1 and AGGTGG as ERRE2). To confirm the exact ERR γ -binding site, we created point mutations into both motifs and tested the construct in comparison to wild-type controls in reporter assays. Reporter activity is decreased in ERRE1- and ERRE1&2-mutated reporter constructs, whereas there is no difference between wt and ERRE2-mutated promoter–reporter constructs (Fig. 4b). Direct binding of ERR γ to the endogenous FGG promoter was proven

by ChIP assays in AML12 cells. Overexpressed ERR γ is recruited to the ERR γ consensus binding site in the FGG promoter, but not to the upstream region lacking an ERRE (Fig. 4c). Finally, we examined the effect of ACEA on the ERRE in the mouse FGG promoter. FGG promoter activity is increased by ACEA, and this effect is blunted when ERRE1-mutated reporter constructs are used (Fig. 4d), suggesting that ACEA-dependent FGG promoter activation is mediated by ERR γ . Overall, these data suggest that activation of the hepatic CB1 receptor induces ERR γ binding to the FGG promoter.

High-fat diet induces ERRγ and fibrinogen gene expression in mice

To clarify the relationship between fibrinogen and obesity in vivo, mice were fed a HFD. H&E and Oil Red O staining

Fig. 2 Overexpression of ERR γ induces fibrinogen expression in mice. **a, b** Mouse primary hepatocytes isolated from wild-type mice infected with Ad-GFP or Ad-ERR γ . Fibrinogen and ERR γ protein levels were measured by western blot. Cell culture medium was collected for the measurement of fibrinogen level. * $p < 0.05$, ** $p < 0.01$ by Student's *t* test. qPCR (c) and western blot (d) analyses were performed to measure mRNA and protein levels in liver. Ad-GFP or Ad-ERR γ were injected into the tail vein of male C57BL/6J mice ($n = 3-4$ per group); mice were killed on day 3. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney *U* and Kruskal-Wallis tests

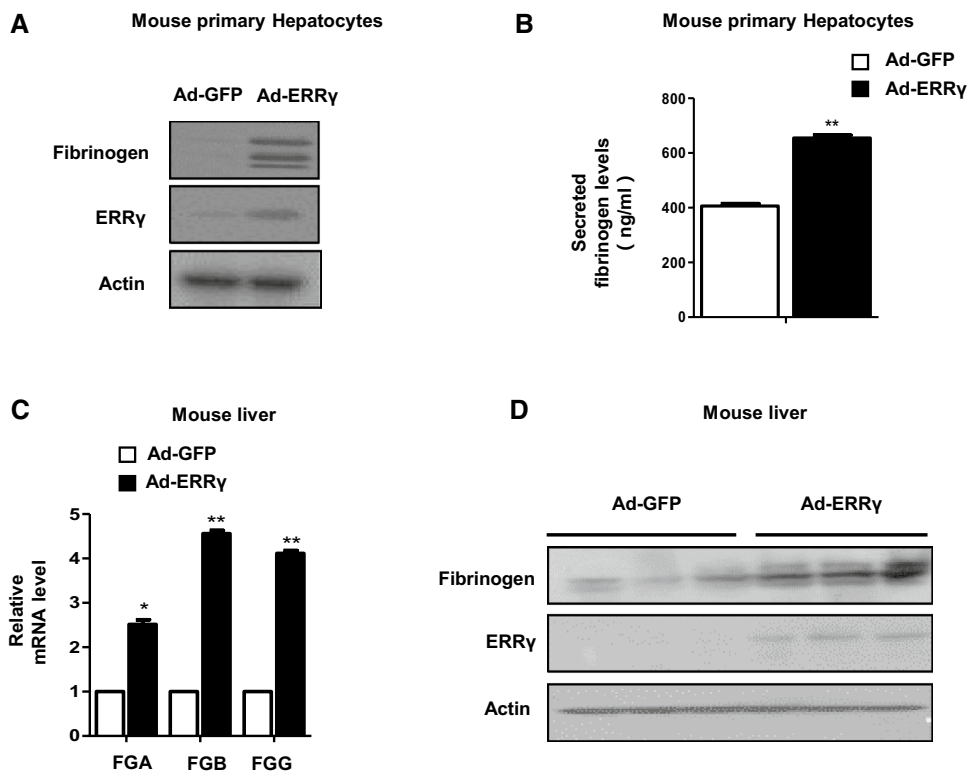
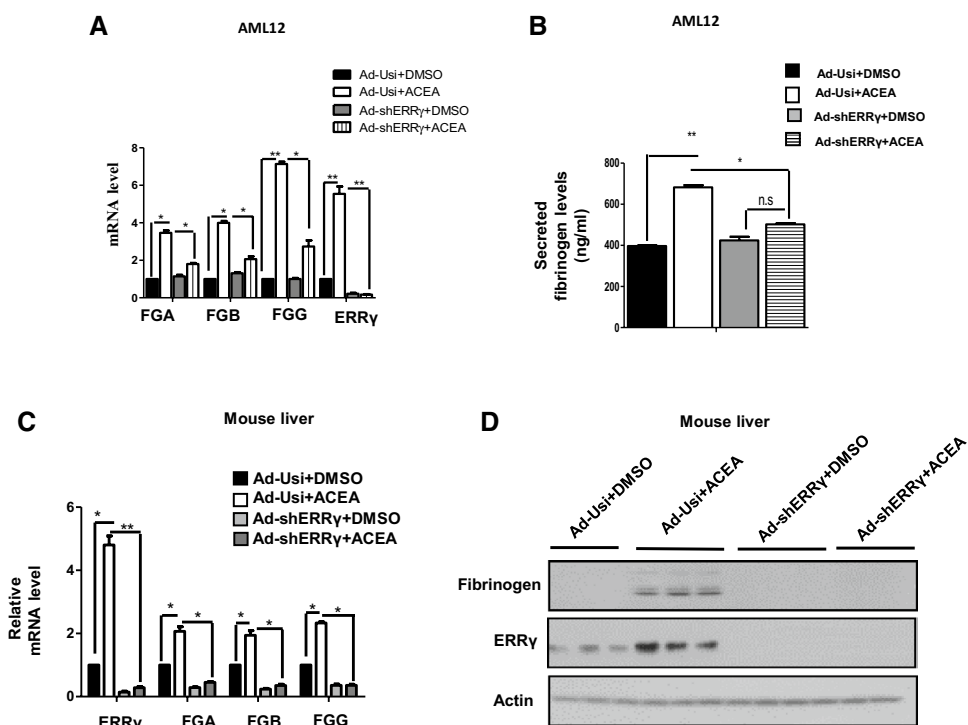


Fig. 3 Knockdown of ERR γ reverse ACEA-mediated induction of fibrinogen expression in mice liver. **a, b** qPCR showing mRNA levels of ERR γ and fibrinogen in AML12 infected with adenovirus US (Ad-US) or adenovirus ERR γ (Ad-shERR γ) in the presence or absence of ACEA. * $p < 0.05$, ** $p < 0.01$ by Student's *t* test. Cell culture medium was collected for the measurement of fibrinogen level. qPCR (c) and western blot (d) analyses showing mRNA and protein levels of hepatic ERR γ and fibrinogen expression in mice which were injected with adenovirus US (Ad-US) or adenovirus ERR γ (Ad-shERR γ) in the presence or absence of ACEA. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney *U* and Kruskal-Wallis tests



of liver sections from these mice show a marked increase in lipid deposition, as expected (Fig. 5a) Semi-quantitative

estimation of hepatic ERR γ and fibrinogen by immunohistochemistry revealed a significant increase of both in livers

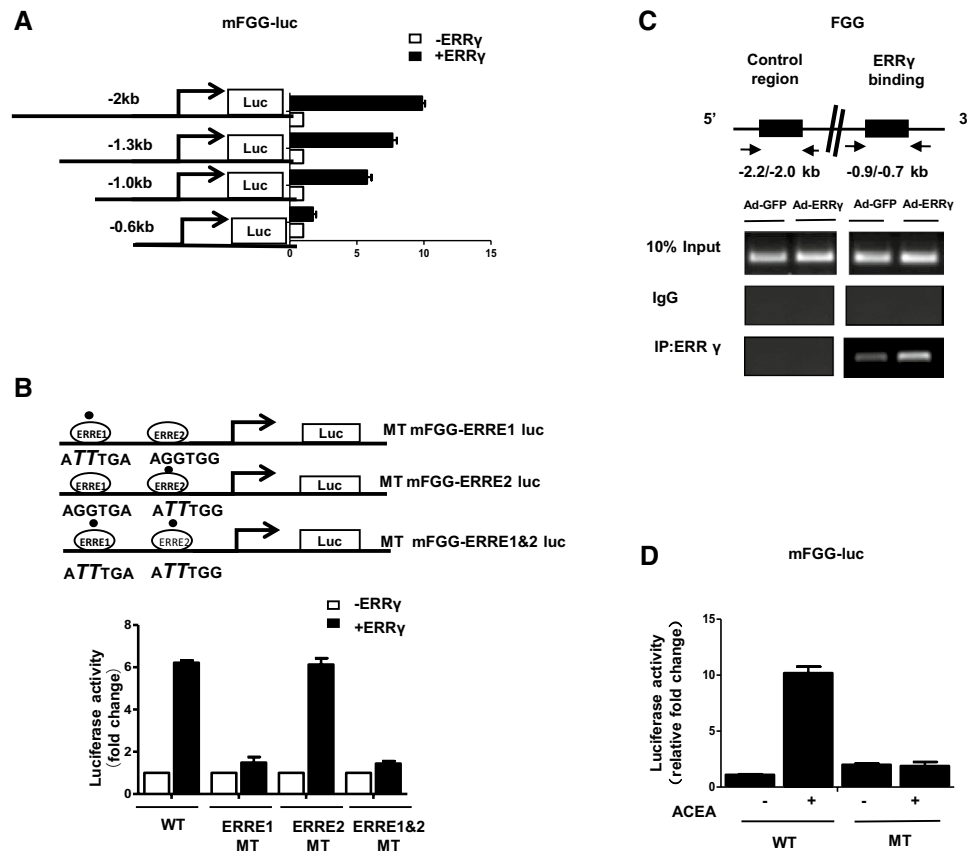


Fig. 4 ERR γ activates the mouse FGG gene promoter. **a** ERR γ induction of mouse FGG promoter activity. AML12 cells were transfected with several deletion constructs of mouse FGG-luc and ERR γ . **b** ERRE-dependent activation of the mouse FGG promoter. AML12 cells were transiently transfected with pCDNA3-FLAG-ERR γ , mFGG-luc (WT), mFGG-Luc (MT ERRE1, MT ERRE2 and MT ERRE1&2). **c** Chromatin immunoprecipitation (ChIP) assay. AML12 cells were infected with Ad-GFP or Ad-ERR γ for 48 h. Input represents 10% of purified DNA in each sample. Cell extracts were immu-

noprecipitated with anti-ERR γ antibody, and purified DNA samples were subjected to PCR with primers targeting the ERRE (– 0.9 to – 0.7 kb) and distal site (– 2.2 to – 2.0 kb) on the mouse fibrinogen promoter. **d** AML12 cells were transfected with mFGG-luc (WT) or mFGG-luc (MT ERRE1). At 36 h post-transfection, cells were treated with ACEA (10 μ M). Cell lysates were subjected to luciferase and β -galactosidase assays. The experiment was conducted in triplicate, and data are expressed as fold activation relative to the control

of HFD mice as compared to control animals fed a NCD (Fig. 5b). These results suggest that increased fibrinogen levels are related to overexpression of ERR γ in diet-induced obese mice.

Knowing that the endocannabinoid system is hyperactivated in obesity stages (Matias et al. 2006) and due to our previous study, where we show that CB1R induces expression of ERR γ and its effect on modulating fibrinogen levels in human hepatocytes (Zhang et al. 2017), we speculated that CB1R might also be the sensor that directs the signal from HFD stress towards fibrinogen expression in liver of HFD mice. Indeed, mRNA and protein levels of the fibrinogen subunits and ERR γ are higher in HFD mice

than in NCD mice (Fig. 5c, d) and these levels are significantly lower in CB1R-KO mice fed a HFD. Moreover, CB1R KO-HFD mice display lower blood fibrinogen levels than WT-HFD mice (Fig. 5e). Together, these results suggest that DIO regulates fibrinogen gene expression via the hepatic CB1R signaling pathway in obese mice.

GSK5182 inhibits CB1 receptor-induced fibrinogen expression and fibrinogen secretion

GSK5182 is an ERR γ inverse agonist that selectively inhibits transactivation of ERR γ (Chao et al. 2006). To further clarify the role of ERR γ in CB1R-mediated induction of

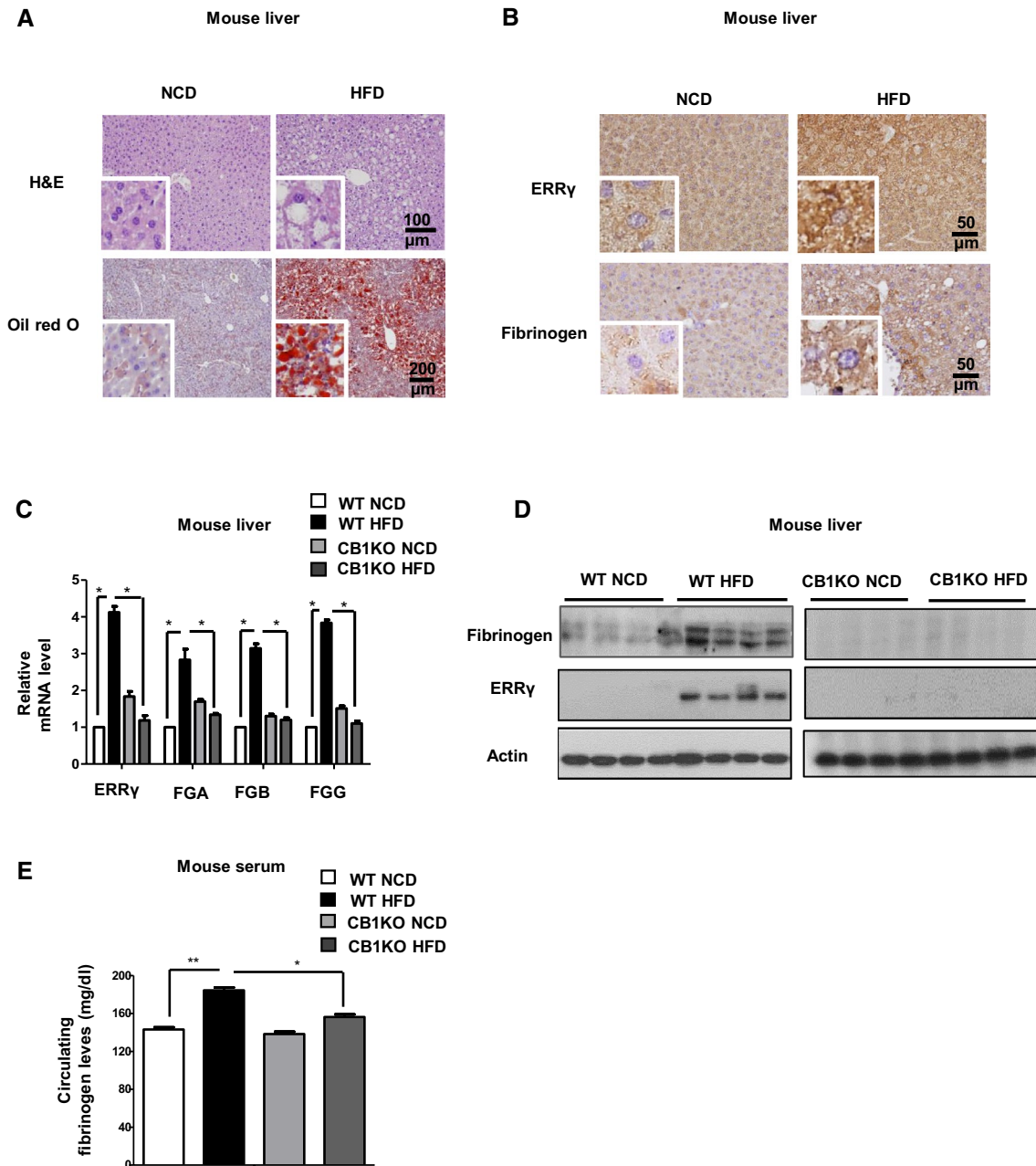


Fig. 5 High-fat diet induces hepatic $ERR\gamma$ and fibrinogen gene expression via CB1R in mice. **a, b** Oil red O staining was performed from the liver samples of mice on a normal chow diet (NCD) or high-fat diet (HFD). IHC analysis showing fibrinogen and $ERR\gamma$ protein expression in liver of mice on a normal chow diet (NCD) or high-fat diet (HFD) ($n=4$). **c–e** Wild-type and CB1R-KO mice maintained

on NCD or HFD. Total RNA for qPCR analyses (**c**) and protein for western blotting (**d**) were extracted from liver tissue. Blood was used to measure fibrinogen level (**e**). All data are representative of at least three independent experiments. Error bars show standard deviation (SD). * $p < 0.05$, by Mann–Whitney U and Kruskal–Wallis tests

fibrinogen gene expression, AML12 cells were transfected with a fibrinogen promoter reporter construct and treated with ACEA in the presence or absence of GSK5182. ACEA-induced mouse FGγ promoter activity (Fig. 6a) and fibrinogen gene expression (Fig. 6b) are inhibited by GSK5182. Consistent with the change in fibrinogen mRNA level,

ACEA-induced fibrinogen protein levels are also significantly decreased by GSK5182 in AML12 cells (Fig. 6c). In addition, fibrinogen levels in culture medium of AML12 cells are dramatically increased upon ACEA treatment, and this increase is significantly attenuated by GSK5182 treatment (Fig. 6d).

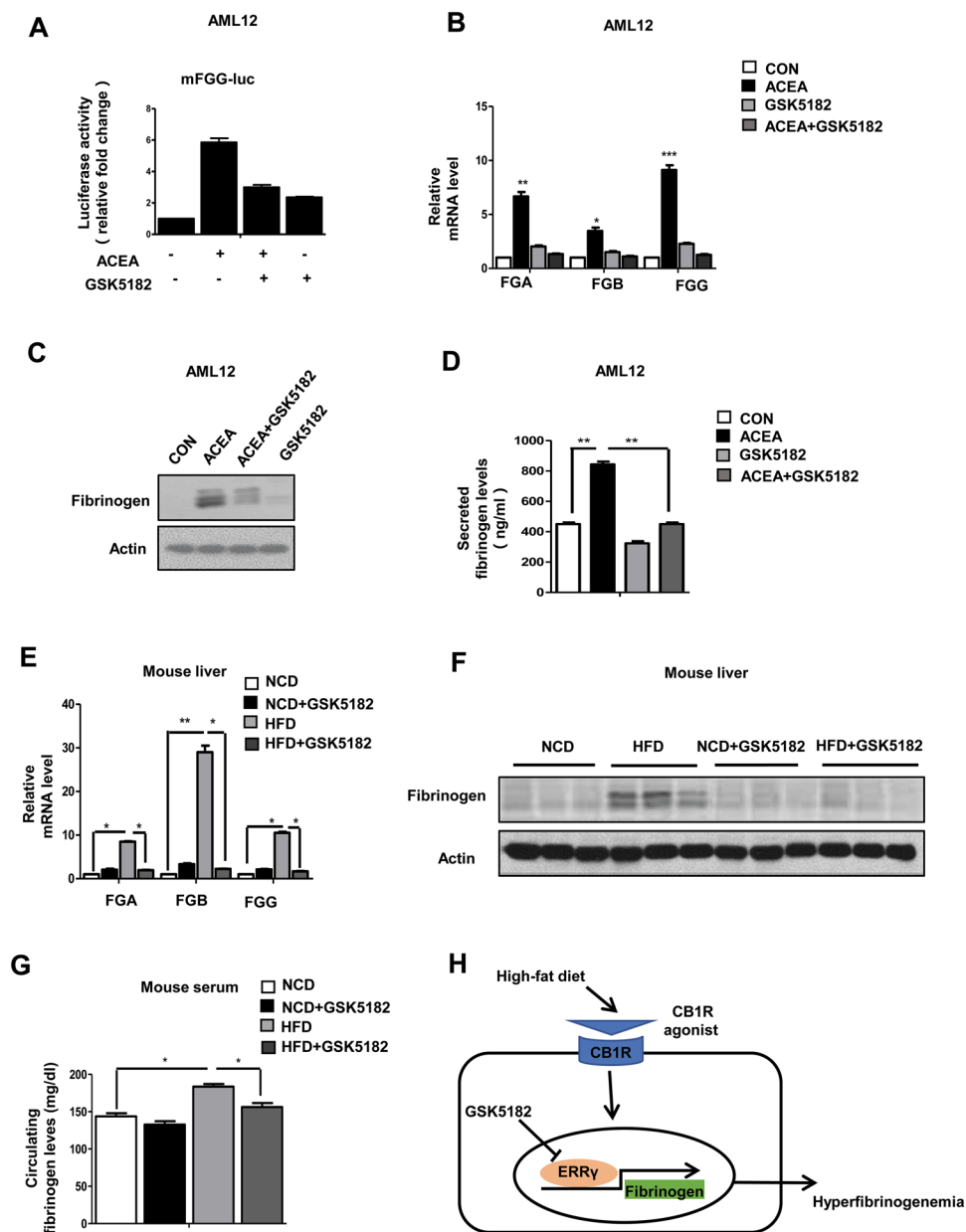


Fig. 6 GSK5182 inhibits fibrinogen gene expression in HFD mice. **a** GSK5182 decreased ACEA-mediated FGG promoter activity. AML12 cells were transfected with vectors expression mFGG-luc, and then treated with ACEA (10 μ M) and/or GSK5182 (10 μ M). **b–d** GSK5182 inhibited ACEA-mediated fibrinogen expression and secretion in AML12 cells. AML12 cells were treated with ACEA (10 μ M) for 12 h. The cell culture medium was replaced, and GSK5182 (10 μ M) was added for the final 24 h. Total mRNA and protein were extracted for qPCR (**b**) and western blot analyses (**c**). Cell culture media were collected to determine fibrinogen levels (**d**). * p <0.05, ** p <0.01, *** p <0.001 by Student's *t* test. **e–g** Mice (n =4) were fed with NCD or HFD for 15 weeks and adminis-

tered with GSK5182 as explained in “Materials and methods”. Total RNA and protein extracted for qPCR (**e**) and western blot (**f**) from liver tissue and blood were used to determine fibrinogen levels (**g**) * p <0.05, ** p <0.01 by Mann–Whitney *U* and Kruskal–Wallis tests. All data are representative of at least three independent experiments. Error bars show SEM. **h** Proposed model for CB1 receptor-mediated induction of fibrinogen gene expression via ERR γ . Activation of hepatic CB1 receptor increases ERR γ gene expression, which in turn increases the promoter activity and mRNA level of fibrinogen. Elevated fibrinogen gene expression causes hyperfibrinogenemia. GSK5182, an ERR γ inverse agonist, inhibits ERR γ -mediated induction of fibrinogen gene expression

Based on the inhibitory effect of GSK5182 on hepatic fibrinogen gene expression in AML12 cells, we assessed fibrinogen expression in GSK5182-treated HFD mice. We, therefore, fed C57BL/6J wild-type mice with a HFD for 15 weeks, which results in increased fibrinogen mRNA levels in liver (Fig. 6e). Intraperitoneal injection of GSK5182 (40 mg/kg/day) for 25 days significantly attenuates HFD-induced fibrinogen mRNA in liver (Fig. 6e). Consistent with mRNA levels, GSK5182 also decreases fibrinogen protein levels in HFD mice (Fig. 6f). In addition, blood fibrinogen levels are lower in the GSK5182-treated group than in control HFD mice (Fig. 6g). These results suggest that HFD induces fibrinogen expression in liver, and that GSK5182 inhibits this induction. Taken together, we show that ERR γ inverse agonist GSK5182 inhibits CB1R-mediated fibrinogen expression and secretion in mice.

Discussion

High circulating fibrinogen levels are associated with an increased risk for myocardial infarction and stroke. Therefore, factors that down-regulate fibrinogen expression may be of importance in the prevention of cardiovascular diseases. In the current study, activation of the hepatic CB1R by ACEA induces ERR γ and fibrinogen gene expression in mice. ERR γ activates not only human but also mouse fibrinogen gene promoter. Remarkably, knock out of CB1R or knockdown of ERR γ inhibits ACEA-mediated induction of fibrinogen gene expression. Finally, GSK5182 inhibits fibrinogen gene expression in HFD mice.

Our studies on the regulatory mechanisms of fibrinogen expression have focused on the fibrinogen γ -chain gene because of findings from most animal and cell culture studies, indicating that the fibrinogen γ chain gene expression is higher compared with α - and β -chains. However, the possibility cannot be excluded that under certain experimental conditions or in different species, regulation of fibrinogen α and β genes are equally important. For example, we observed that fibrinogen- α expression is much higher than β and γ , upon overexpressing ERR γ in human hepatoma cell line Huh7 cells (Zhang et al. 2017), whereas ERR γ overexpression brings up FGB and FGG expression in Ad-GFP-injected mice liver samples (Fig. 2c).

Fibrinogen is a “positive” acute-phase protein, i.e., its blood levels rise in response to systemic inflammation, tissue injury and certain other events. It is also elevated in various cancers. One of the key leukocyte-derived APR (acute phase response) cytokines, IL-6, stimulates a coordinated increase in fibrinogen mRNA and protein expression, with the majority of regulation occurring at the transcriptional level (Fuller et al. 1985; Ritchie and Fuller 1983). The APR

rapidly changes plasma levels of multiple liver-derived proteins, thus priming the response to injury and infection (Gabay and Kushner 1999). Our groups’ previous results suggest that *S. typhimurium*-mediated IL-6 induction affects expression of hepatic ERR γ and hepcidin (Kim et al. 2014). In line with these published data, we found that treatment of mouse and human hepatocytes with IL-6 induced fibrinogen mRNA and protein levels, and those could be significantly reduced following knockdown of ERR γ (data not shown). Additional studies are necessary to examine the role of ERR γ in IL-6-mediated fibrinogen gene expression in future.

Non-alcoholic fatty liver disease (NAFLD) has been traditionally regarded as the consequence of a high-fat western diet and sedentary lifestyle (Browning et al. 2004; Feldstein 2010). Cardiovascular disease (CVD) remains one of the major causes of excess mortality in NAFLD patients (Adams et al. 2005; Browning et al. 2004; Harrison and Day 2007). Regarding liver, accumulating evidence indicates that the cannabinoid system plays a crucial role in the pathophysiology of many liver diseases, both as a key player in hepatic injury and as a mediator of complications of cirrhosis. Previous reports show that CB1R signaling in peripheral tissues contributes to the metabolic syndrome (Osei-Hyiaman et al. 2005, 2008b), raising the possibility that selectively targeting CB1R in peripheral tissue may be a promising therapeutic approach. Such concept was supported by recent studies, where a peripherally restricted neutral CB1 antagonist AM6545 (Tam et al. 2010) or the peripheral CB1 inverse agonists JD5037 (Tam et al. 2012) ameliorated diet-induced obesity in mice under high-fat diet. Our own recent study suggests that patients with NAFLD have increased blood fibrinogen levels (Zhang et al. 2017). In this study, our results further proved that ERR γ -induced hyperfibrinogenemia is inhibited by depleting CB1R in HFD mice (Fig. 5). Therefore, CB1 receptors should be further assessed as a potential therapeutic target in NAFLD caused CVD, and blunting the CB1R signaling pathway should be as well important to reduce fibrinogen expression.

Previous research with a 14-day HFD rat model suggested that there are significant and positive correlations between plasma fibrinogen levels and serum lipid content, and between fibrinogen levels and VLDL–LDL–lipid content. Clinofibrate, in addition to its beneficial effect on the serum lipid profile, can effectively reduce the plasma fibrinogen level (Okazaki et al. 1994). Many oral drugs have also been shown to lower fibrinogen. Among the oral fibrinogen-lowering drugs, fibrates rank first (e.g., bezafibrate has been reported to reduce increased fibrinogen by as much as 40%, and ticlopidine can induce a reduction of about 15%, if fibrinogen is elevated at baseline) (Kockx et al. 1999). Our current study also demonstrates that fibrinogen gene expression is decreased in a GSK5182-treated HFD mouse model (Fig. 6). GSK5182 is currently one of the leading ERR γ

inverse agonists and shows promise in the development of drugs targeting ERR γ -related diseases.

Overall, our results reveal that ERR γ plays a significant role in the CB1 receptor-mediated fibrinogen gene expression in vivo. When taking a high-fat diet, CB1R signaling is activated following induction and activation of ERR γ . GSK5182, an ERR γ inverse agonist markedly reduces ERR γ -dependent fibrinogen gene expression (Fig. 6h). Therefore, targeting ERR γ with GSK5182 can be of therapeutic potential to ameliorate hyperfibrinogenemia in vivo.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The manuscript does not contain clinical studies or patient data.

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