Archives of Toxicology (2020) 94:427–438
https://doi.org/10.1007/s00204-019-02648-7

MOLECULAR TOXICOLOGY

An inverse agonist of estrogen-related receptor γ regulates 2-arachidonoylglycerol synthesis by modulating diacylglycerol lipase expression in alcohol-intoxicated mice

Yoon Seok Jung[1] · Yong-Hoon Kim[2,3] · Kamalakannan Radhakrishnan[1] · Jina kim[4] · Don-Kyu Kim[1] · Ji-Hyeok Lee[5] · Hyunhee Oh[5] · In-Kyu Lee[6,7] · Wook Kim[8] · Sung Jin Cho[4,7] · Cheol Soo Choi[5,9] · Steven Dooley[10] · Josephine M. Egan[11] · Chul-Ho Lee[2,3] · Hueng-Sik Choi[1]

Received: 6 September 2019 / Accepted: 12 December 2019 / Published online: 7 January 2020

Abstract

Chronic alcohol feeding increases the levels of 2-arachidonoylglycerol (2-AG) in the liver, which activates hepatic cannabinoid receptor type 1 (CB1R), leading to oxidative liver injury. 2-AG biosynthesis is catalyzed by diacylglycerol lipase (DAGL). However, the mechanisms regulating hepatic DAGL gene expression and 2-AG production are largely unknown. In this study, we show that CB1R-induced estrogen-related receptor γ (ERRγ) controls hepatic DAGL gene expression and 2-AG levels. Arachidonyl-2′-chloroethylamide (ACEA), a synthetic CB1R agonist, significantly upregulated ERRγ, DAGLα, and DAGLβ, and increased 2-AG levels in the liver (10 mg/kg) and hepatocytes (10 μM) of wild-type (WT) mice. ERRγ overexpression upregulated DAGLα and DAGLβ expressions and increased 2-AG levels, whereas ERRγ knockdown abolished ACEA-induced DAGLα, DAGLβ, and 2-AG in vitro and in vivo. Promoter assays showed that ERRγ positively regulated DAGLα and DAGLβ transcription by binding to the ERR response element in the DAGLα and DAGLβ promoters. Chronic alcohol feeding (27.5% of total calories) induced hepatic steatosis and upregulated ERRγ, leading to increased DAGLα, DAGLβ, or 2-AG in WT mice, whereas these alcohol-induced effects did not occur in hepatocyte-specific CB1R knockout mice or in those treated with the ERRγ inverse agonist GSK5182 (40 mg/kg in mice and 10 μM in vitro). Taken together, these results indicate that suppression of alcohol-induced DAGLα and DAGLβ gene expressions and 2-AG levels by an ERRγ-specific inverse agonist may be a novel and attractive therapeutic approach for the treatment of alcoholic liver disease.

Keywords 2-AG · Hepatic CB1R · DAGL · Endocannabinoid · ERRγ · GSK5182

Introduction

Alcoholic liver disease (ALD) is a severe medical complication of alcohol abuse and the primary cause of chronic liver disease in the Western world (Gao and Bataller 2011). Chronic alcohol drinking leads to the development of fatty liver, which can progress to steatohepatitis and liver cirrhosis (Dey and Cederbaum 2006; Garcia-Villafranca et al. 2008; Lieber and Schmid 1961; Lieber et al. 1966; You et al. 2002). Alcohol drinking increases lipogenesis and decreases fatty acid oxidation in the liver (Garcia-Villafranca et al. 2008; You et al. 2004). Moreover, chronic alcohol use promotes the production of reactive oxygen species (ROS) such as superoxide or hydrogen peroxide, which increase oxidative stress in the liver (Arteel 2003; Cederbaum et al. 2009). Alcohol is oxidized to acetaldehyde through the action of alcohol dehydrogenase and the cytochrome P450-dependent microsomal ethanol oxidizing system (MEOS) (Cederbaum et al. 2009). Cytochrome P450 2E1 (CYP2E1), a key enzyme of MEOS, is a crucial mediator of alcohol-induced ROS and liver injury (Gonzalez 2005).

Endogenous cannabinoids (CB; endocannabinoids) are lipid mediators that interact with two G protein-coupled CB receptors, CB1 and CB2, taking part in a complex lipid signaling network. The CB1R is expressed in brain, vascular tissues, heart and liver. The CB2 receptor is expressed

Yoon Seok Jung and Yong-Hoon Kim contributed equally to this work.

✉ Chul-Ho Lee
chullee@kribb.re.kr

✉ Hueng-Sik Choi
hsc@chonnam.ac.kr

Extended author information available on the last page of the article

in most immune and hematopoietic cells. Arachidonoyl ethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) are endogenous cannabinoid activators of CB1 and CB2 (Sugiura et al. 2002). 2-AG is synthesized from arachidonic acid-containing membrane phospholipids through the action of phosphatidylinositol (PI)–phospholipase C (PLC) and diacylglycerol (DAG) lipase (DAGL). 2-AG synthesis involves the sequential hydrolysis of PI catalyzed by (PI)-phospholipase A_1 (PLA_1) and lyso PI-specific PLCs, and is regulated by the hydrolysis of DAG by DAGLα and DAGLβ (Ueda et al. 2011). However, the mechanism underlying the regulation of hepatic DAGLα and DAGLβ gene expressions remains unknown.

The estrogen-related receptor (ERR) subfamily consists of three members, ERRα, β, and γ (also known as NR3B1–3). ERRs bind to both classic estrogen response elements as dimers or to the half-site core sequence (TNAAGGTCA; ERR response element or ERRE) as monomers. ERRs are expressed at high levels in tissues with a high metabolic demand and are regulated by a peripheral circadian clock in key metabolic tissues such as white and brown adipose tissues, muscle, pancreas, heart, brain, and liver (Giguere et al. 1988; Luo et al. 2003; Razzaque et al. 2004). Structural studies suggest that ERRγ is constitutively active in the absence of endogenous ligands, whereas small molecule ligands activate or repress ERRγ transactivation (Giguere 2008). GSK5182, a specific inverse agonist of ERRγ, inhibits its transcriptional activity by recruiting small heterodimer partner (SHP)-interacting leucine zipper protein (SMILE) (Xie et al. 2009). The ligand-independent transactivation of ERRγ is regulated by nuclear receptor co-regulators such as PGC-1α, NCoA-2, receptor-interacting protein 140 (RIP140), SMILE, and SHP, which are involved in liver metabolism (Hentschke et al. 2002; Herzog et al. 2007; Hong et al. 1999; Huss et al. 2002; Sanyal et al. 2002). ERRγ transcriptional activity is inhibited by protein kinase B/Akt-mediated phosphorylation at S179 and translocation of ERRγ from the nucleus to the cytoplasm (Kim et al. 2014b). ERRγ induces gluconeogenesis by regulating the expression of glucose-6-phosphate and phosphoenolpyruvate carboxykinase 1 (Kim et al. 2012). We recently showed that ERRγ increases bile acid synthesis by inducing CYP7A1 gene expression (Zhang et al. 2015), and the ERRγ inverse agonist GSK5182 controls hepcidin gene expression and improves salmonella typhimurium infection by modulating host iron homeostasis (Kim et al. 2014a). We also reported that ERRγ induces pyruvate dehydrogenase kinase 4 gene expression (Lee et al. 2012). Furthermore, hepatic ERRγ regulates Lipin-1 gene expression and DAG levels in hepatocytes (Kim et al. 2011).

In this study, we demonstrate that ERRγ is required for CB1R-induced DAGLα and DAGLβ gene expressions and 2-AG synthesis in hepatocytes.

Materials and methods

Animal studies

Male mice were used for all experiments. C57BL/6 wild-type (WT) mice were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, Korea). $CNR1^{flox/flox}$ mice were generated as described previously (Gonzalez-Mariscal et al. 2018). Mice expressing Cre under the Alb gene promoter (Alb1-Cre, stock # 016832; Jackson Laboratories, Bar Harbor, Maine) and $CNR1^{flox/flox}$ mice were bred to obtain $CNR1^{flox/flox}$-Alb-Cre^{+} (CB1R HKO) mice and $CNR1^{flox/flox}$-Alb-Cre^{-} (WT) control littermates. Prior to experiments, mice were acclimatized to a 12 h light/dark cycle at 22 ± 2 °C for 2 weeks with unlimited food and water in a specific pathogen-free facility. To setup the CB1 receptor agonist treatment model, 8-week-old WT mice were treated with ACEA (10 mg/kg) for the indicated days. Ad-GFP or Ad-ERRγ was injected into male C57BL/6 J mice via the tail vein. Mice were sacrificed 5 days after adenovirus injection. Ad-US and Ad-shERRγ were injected into male C57BL/6J mice via the tail vein. Four days after injection, mice were treated with ACEA (10 mg/kg) for 3 days. GSK5182 (40 mg/kg) was administered to male C57BL/6J mice daily by intraperitoneal injection for 4 days. ACEA (10 mg/kg) was also given daily by intraperitoneal injection during the final 3 days. To establish the chronic alcoholic hepatosteatosis model, 8-week-old WT and CB1R HKO mice were treated for 4 weeks with an alcohol-containing Lieber–DeCarli formulation-based liquid (Dyets, Bethlehem, PA, USA) diet (27.5% of total calories) or a pair-fed control liquid diet in which alcohol was replaced isocalorically with carbohydrate. For the compound study, mice were assigned to four groups: (a) alcohol-containing Lieber–DeCarli formulation-based liquid diet, (b) pair-fed control diet in which alcohol was replaced isocalorically with carbohydrate, (c) control diet supplemented with GSK5182 (40 mg/kg, p.o.), and (d) alcohol-containing diet supplemented with GSK5182. In the latter two groups, GSK5182 was injected once daily for the last 2 weeks of the study. All mice were euthanized by CO_2 asphyxiation. All experimental procedures were approved by the Institutional Animal Care and Use Committee of KRIBB (Daejeon, Korea), and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Guide for the Care and Use of Laboratory Animals, 8th edition).

Chemicals

GSK5182 was synthesized as described previously (Chao et al. 2006) and used at a concentration of 40 mg/kg for

in vivo experiments and 10 μM for in vitro experiments. Arachidonyl-2′-chloroethylamide (ACEA) was purchased from Tocris Bioscience.

Histopathology

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 μm using a cryotome (Sakura Finetek). Cryostat sections of liver tissue were fixed in 10% neutral buffered formalin. After fixation, liver tissue sections were stained with 0.3% oil-red-O solution and counterstained with hematoxylin. Images were captured using a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

Plasmids and DNA constructs

The promoters of mouse DAGLα (− 2487 bp/ + 360 bp) and DAGLβ (− 2966 bp/ + 35 bp) were cloned into the SacI/BglIII site of the PGL3-basic vector. DAGLα ERR response element mut-Luc ($^{-763bp}$TCAGGTCACA^{-753bp} to $^{-763bp}$TCATTTCACA^{-753bp}) and DAGLβ ERR response element mut-Luc ($^{-1368bp}$CCAGGTCACT$^{-1358bp}$ to $^{-1368bp}$CCATTTCACT$^{-1358bp}$) were generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). FLAG-ERRα, FLAG-ERRβ, and FLAG-ERRγ constructs were described previously (Sanyal et al. 2002). All plasmids used were confirmed by complete sequence analysis.

Recombinant adenoviruses

Ad-GFP, Ad-FLAG-ERRγ, Ad-US, and Ad-shERRγ were described previously (Xie et al. 2009). All viruses were purified by $CsCl_2$. Adenovirus infection in cells and mice was performed as described previously (Xie et al. 2009).

Cell culture and transient transfection assays

HepG2 (human hepatoma cells), 293T (human embryonic kidney cells), and AML12 (mouse immortalized hepatocytes) cells were obtained as described previously (Ryu et al. 2009). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and used for experiments at 75% confluence. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions/protocol. The cells were treated with 10 μM GSK5182 unless noted otherwise. After 48 h of transfection, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity.

RNA isolation and analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and real-time quantitative PCR (qPCR) analysis was performed using the following primers: ERRγ (mouse/human), 5′-AAGATCGACACATTGATTCCAGC-3′ (forward) and 5′-CATGGTTGAACTGAATTCCCAC-3′ (reverse); DAGLα (mouse), 5′-CTGTCTGTGGTGCTCTTCG-3′ (forward) and 5′-CGGGGTTCTGTGTAGAGGA-3′ (reverse); DAGLα (human), 5′-CGGCCTGGTCTATAACCCG-3′ (forward) and 5′-ATCTCAGCGATCATGCAGCTC-3′ (reverse); DAGLβ (mouse), 5′-GGGAAGATGGCTCCGTATC-3′ (forward) and 5′-ACGCCACCCTGGTGTTATC-3′ (reverse); DAGLβ (human), 5′-ATGCCGGGGATGGTACTCTT-3′ (forward) and 5′-CAGAATGCCAATCCACCACAG-3′ (reverse); and CB1R (mouse), 5′-GATCTTAGACGGCCTTGCAG-3′ (forward) and 5′-TTGGATGCCATGTCTCCTTT-3′ (reverse). Data were normalized to β-actin (mouse/human) expression, which was determined using 5′-TCTGGCACCACACCTTCTAC-3′ (forward) and 5′-TCGTAGATGGGCACAGTGTGG-3′ (reverse) primers.

Western blot analysis

Cultured cells or mouse liver tissues were lysed with RIPA buffer and subjected to immunoblot analysis as described previously (Lee et al. 2008). The membranes were probed with anti-ERRγ (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000), anti-β-actin (AbFrontier, Seoul, Korea; diluted 1:5000) (Kim et al. 2013), and anti-DAGLα (Cell Signaling Technology; 1:1000) (Martin et al. 2016) antibodies.

Analysis of 2-AG by gas chromatography–mass spectrometry (GC–MS)

Samples were extracted with 1 mL cold acetonitrile (ACN) in a tissue lyser with an iron ball for 1 min. The homogenized sample was transferred to a 7 mL vial containing 3 mL ACN. The homogenized sample was centrifuged, and the supernatant was extracted and dried using N_2 gas (37 °C). Dried extracts (1 mg in a GC vial) were treated with 70 μL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) (99:1), heated at 60 °C for 30 min, and allowed to cool to room temperature. The derivatized samples were analyzed by GC–MS (Agilent 7890 Series GC system, Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5975C mass spectrometer (Agilent Technologies) using the HP-5MS capillary column (30 m × 0.20 mm i.d. × 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA), and run under

the following GC temperature program: initial 80 °C held for 5 min, raised to 240 °C at 20 °C /min rate, held at this temp for 8 min, then raised to 320 °C at 40 °C/min rate, and finally held at this temperature for 5 min. The injection port, GC interface, and ionization chamber were maintained at 270 °C, 230 °C, and 150 °C, respectively. Ultra-high purity helium was used as the carrier gas and the flow rate was 2 mL/min. The sample injection volume was 3 µL. The MS detector was a magnetic sector; spectra were acquired in the positive, low resolution, total ion scan mode, and selected ion mode (203 m/z for 2-AG). The retention time for 2-AG was 21.3 min. Quantitative determination of 2-AG in the samples was performed using a calibration curve from an external standard of 2-AG (9,179,881.51x − 1,348,658.11; R2 = 1.00).

Lipid quantification

Frozen livers were homogenized in 0.9% saline, and a 100% chloroform:100% methanol (1:2, v/v) solution was then added to the samples. The solution was vortexed and centrifuged at 890×g for 20 min. The chloroform layer was transferred to a glass tube and air-dried until only the pellet remained. Hepatic triglyceride (TG) concentration was analyzed using a commercially available kit (cat. no. AM 157S-K; Asan Pharmaceuticals Co., Ltd., Seoul, Korea) in accordance with the manufacturer's protocol.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). Immunoprecipitation was performed using an anti-ERRγ antibody or IgG as a negative control. After recovery of DNA, qPCR was performed using primers encompassing the DAGL-α and -β promoter regions. The primers used for PCR were as follows: DAGLα − 1.9 kb/ − 1.7 kb, 5′-ATGTGAAAGTTTGGGAAATT-3′ (forward) and 5′-GATGCCCTACATGGGGCTCA-3′ (reverse); and − 0.8 kb/ − 0.6 kb, 5′-GCTGCAGCTGCACAGGGGGT-3′ (forward) and 5′-TGGCTACAGTCAATTGCTGG-3′ (reverse); DAGLβ − 2.7 kb/ − 2.5 kb, 5′-AAGAAAATTTACAAAAGATT-3′ (forward) and 5′-GAAGCAGAGTTTAGAAAGCA-3′ (reverse); and − 1.5 kb/ − 1.3 kb, 5′-TCTGGGGTACACTGCTGGGA-3′ (forward) and 5′-ATTAAATAAAATTGGCTACA-3′ (reverse).

Statistical analyses

Numerical data are presented as mean ± SEM. Comparison between two groups was performed using the two-tailed Student's t test, whereas comparison between multiple groups was performed by ordinary one-way ANOVA with Tukey's multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

Results

Activation of CB1R increases DAGLα and DAGLβ gene expressions and 2-AG levels

In previous work, we showed that CB1R signaling induces ERRγ gene expression (Kim et al. 2013). In this study, we hypothesized that CB1R-induced ERRγ regulates hepatic 2-AG synthesis. ACEA, a CB1R-specific agonist, significantly upregulated ERRγ, DAGLα, and DAGLβ mRNA in AML12 at 1 h, and maximum levels were reached at 6 h. In HepG2 cells, ERRγ, DAGLα, and DAGLβ mRNA levels are increased by ACEA treatment at 3 h, reaching maximum levels at 6 h (Fig. 1a). CB1R activation by ACEA significantly increased 2-AG levels in AML12 cells and HepG2 cells (Fig. 1b). In C57BL/6J mice, injection of ACEA significantly increased hepatic ERRγ, DAGLα, and DAGLβ mRNA levels, and maximum levels were observed at 3 days after ACEA injection (Fig. 1c). Western blot analysis shows that ACEA significantly induces ERRγ protein expression in the liver of mice at day 1, which sustaines until day 5. In accordance with changes in ERRγ protein expression, DAGLα protein levels are increased at day 1 after ACEA treatment (Fig. 1d). Finally, ACEA treatment significantly increases 2-AG levels at day 1 after injection, reaching maximum levels at day 5 (Fig. 1e). These data indicate that ACEA increases DAGLα and DAGLβ gene expressions and 2-AG levels.

ERRγ overexpression or knockdown regulate hepatic DAGLα and DAGLβ gene expressions and 2-AG levels

Overexpression of ERRγ using adenoviral ERRγ (Ad-ERRγ) significantly upregulated DAGLα and DAGLβ mRNA expressions in AML12 and HepG2 cells (Fig. 2a). Overexpression of ERRγ also increased 2-AG levels in AML12 and HepG2 cells (Fig. 2b). DAGLα and DAGLβ mRNA levels, DAGLα protein expression, and 2-AG levels were increased in Ad-ERRγ-infected mouse livers (Fig. 2c–e). To determine whether the effect of ACEA on hepatic DAGLα and DAGLβ gene expressions and increased 2-AG levels was ERRγ dependent, ERRγ was knocked down by adenoviral shERRγ (Ad-shERRγ) infection. ERRγ depletion significantly decreased ACEA-induced DAGLα and DAGLβ mRNA expressions, DAGLα protein expression, and 2-AG levels (Fig. 2f–j). These data demonstrate that ERRγ is required for ACEA-induced DAGLα and DAGLβ gene expressions and 2-AG biosynthesis.

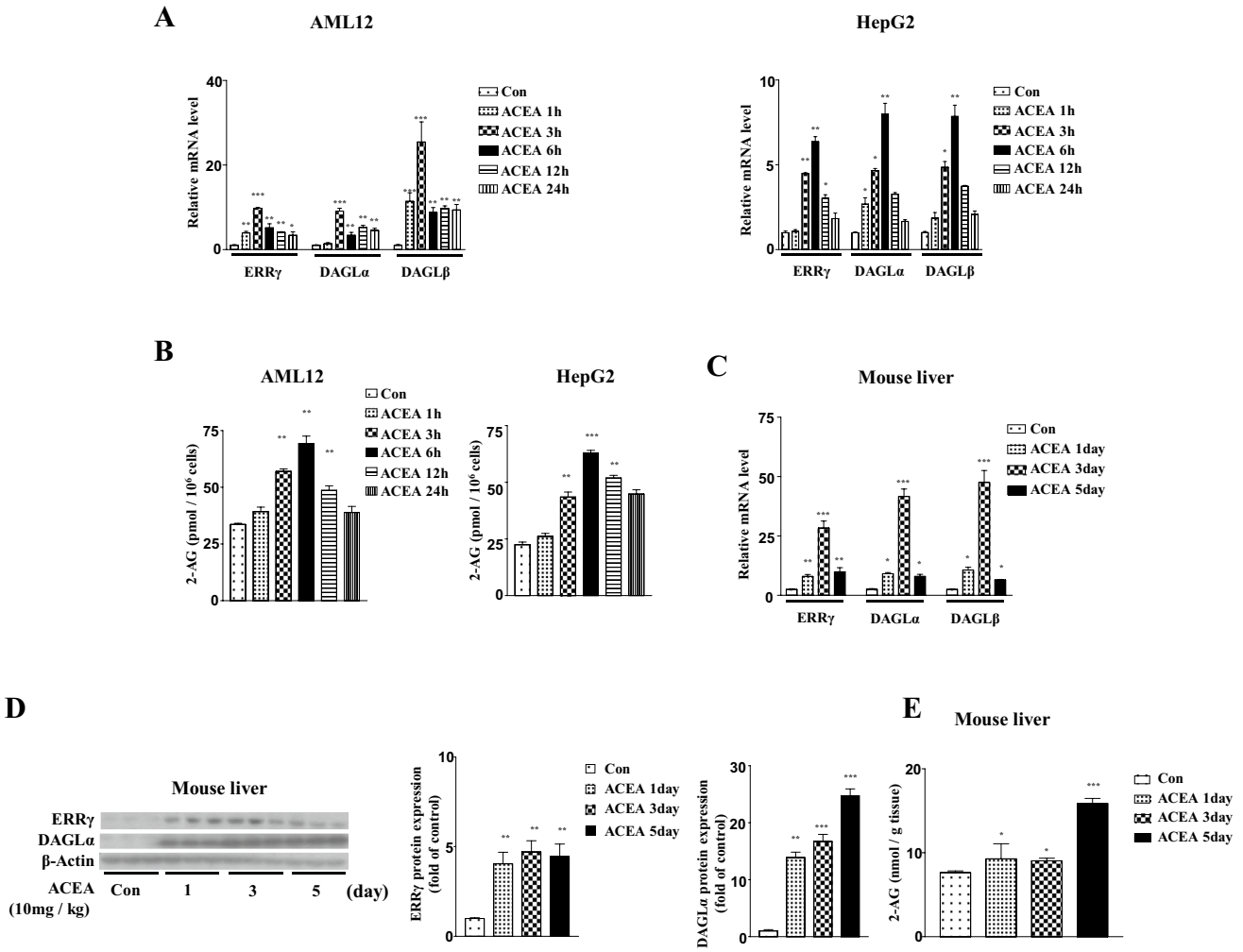


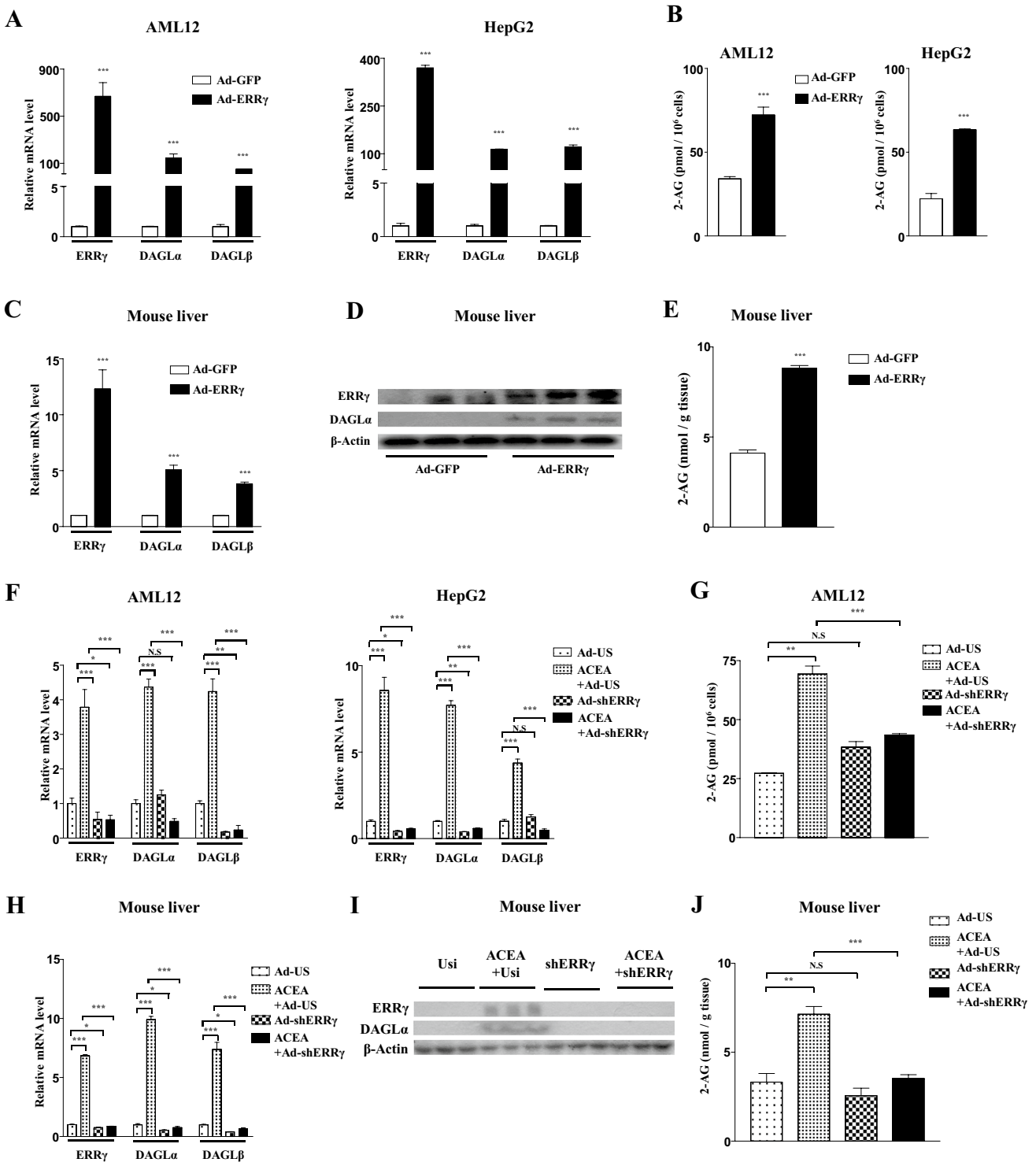
Fig. 1 ACEA induces DAGLα and DAGLβ gene expression and increases 2-AG levels. **a** Q-PCR analysis of total RNAs isolated from AML12 and HepG2 cells treated with ACEA for different times as indicated. **b** Measurement of 2-AG levels from ACEA-treated AML12 and HepG2 cells. **c** Q-PCR analysis of total RNAs isolated from livers of ACEA-treated mice ($n=5$ per group). **d** Western blot analysis of total protein isolated from livers of ACEA-treated mice. ERRγ and DAGLα western blot bands were quantified using ImageJ software and normalized to actin ($n=3$ per group). **e** Measurement of 2-AG levels in livers from ACEA-treated mice ($n=5$ per group). All cell culture experiments were performed as three independent replicates. Data represent mean ± SEM. All data were analyzed by two-tailed Student's *t* test as $*p<0.05$; $**p<0.01$; $***p<0.001$

ERRγ activates DAGLα and DAGLβ gene promoter activity

To examine the direct ERRγ-mediated transcriptional activation of the DAGLα and DAGLβ gene promoters upon ACEA stimulation in AML12 cells, gene promoter-reporter studies were performed after modulating ERRγ expression. Knockdown of ERRγ by Ad-shERRγ decreased, whereas overexpression of ERRγ, but not that of ERRα and ERRβ, increased luciferase activity in AML12 cells in response to ACEA stimulation (Fig. 3a, b). ERRγ overexpression/ACEA-induced reporter activity was significantly inhibited in a mutant reporter construct generated by site-directed mutagenesis of a conserved ERRγ consensus sequence (AGGTCA, a consensus response element) in the DAGLα and the DAGLβ promoters (Fig. 3c–e). ACEA-induced ERRγ binding to the DAGLα and DAGLβ promoters was confirmed using ChIP assays (Fig. 3f, g). Collectively, these data indicate that ERRγ is a direct transcriptional activator of DAGLα and DAGLβ in response to ACEA-mediated CB1 activation.

The ERRγ-specific inverse agonist GSK5182 inhibits ACEA-induced DAGLα and DAGLβ gene expressions and 2-AG levels

GSK5182, an ERRγ-specific inverse agonist, suppresses ERRγ target gene expression by inhibiting ERRγ



transactivation. GSK5182 inhibited ACEA-induced DAGLα and DAGLβ promoter activation in AML12 cells (Fig. 4a). GSK5182 treatment decreased ACEA-induced DAGLα and DAGLβ mRNA levels in AML12 and HepG2 cells (Fig. 4b) and 2-AG levels in AML12 cells (Fig. 4c). GSK5182 administration decreased ACEA-induced DAGLα and β mRNA levels, DAGLα protein, and 2-AG levels in the mouse liver (Fig. 4d, e). These data suggest the potential of GSK5182 as a small molecule inhibitor of the CB1R/2-AG axis.

◂**Fig. 2** ERRγ overexpression and knockdown modulate DAGLα and β gene expressions and 2-AG levels. **a** Q-PCR analysis of total RNAs isolated from AML12 and HepG2 cells infected with Ad-GFP or Ad-ERRγ. **b** Measurement of 2-AG levels in AML12 and HepG2 cells infected with Ad-GFP or Ad-ERRγ. **c** Q-PCR analysis of total RNAs isolated from livers of Ad-GFP- or Ad-ERRγ-infected mice ($n=5$ per group). **d** Western blot analysis of total protein isolated from livers of Ad-GFP- or Ad-ERRγ-infected mice ($n=3$ per group). **e** Measurement of 2-AG levels in livers from Ad-GFP- or Ad-ERRγ-infected mice ($n=5$ per group). Q-PCR analysis of total RNAs **f** and measurement of 2-AG levels **g** in AML12 and HepG2 cells infected with Ad-US or Ad-shERRγ for 36 h, and then treated with ACEA for 3 h. Q-PCR analysis of total RNAs (**h**), western blot analysis of total proteins (**i**) and measurement of 2-AG levels (**j**) in livers of Ad-US- or Ad-shERRγ-infected mice, treated or not with ACEA ($n=4$ per group). All cell culture experiments were performed as three independent replicates. Data represent mean ± SEM. Data in **a**–**c**, **e** were analyzed by two-tailed Student's *t* test, and data in **f**–**h**, **j** were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test as $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; not significant (N.S)

Alcohol feeding-induced DAGLα and DAGLβ gene expressions and 2-AG production are absent in CB1R knockout mice and inhibited upon GSK5182 treatment

In vitro findings were confirmed in a model of alcohol-induced damage generated by exposing hepatocyte-specific CB1R knockout mice to alcohol feeding for 4 weeks. Hepatic steatosis, assessed by oil-red-O staining of liver sections, was significantly lower in ethanol-fed CB1R HKO mice than in WT mice (Fig. 5a). Consistent with these observations, alcohol treatment-induced hepatic TG levels were significantly lower in hepatocyte-specific CB1R depleted mice than in WT mice (Fig. 5b). Hepatocyte-specific CB1R ablation or GSK treatment inhibited the effect of chronic alcohol exposure on upregulating ERRγ, DAGLα, and DAGLβ mRNA and protein and increasing 2-AG levels (Fig. 5c–e). Finally, we tested the effect of GSK5182 on DAGLα and β gene expressions and 2-AG levels in chronic alcohol-fed mice. Chronic alcohol exposure dramatically upregulated DAGLα and DAGLβ mRNA expressions, and GSK5182 inhibited alcohol-induced DAGLα and DAGLβ in the mouse liver (Fig. 5f). DAGLα protein levels and 2-AG levels were also inhibited by GSK5182 in the mouse liver (Fig. 5g, h). These data suggest that chronic alcohol exposure-mediated ERRγ, DAGLα, and DAGLβ upregulation and increase in 2-AG levels were dependent on CB1R activation in hepatocytes and associated with the development of alcoholic fatty liver. GSK5182 inhibited alcohol feeding-induced DAGLα and DAGLβ gene expressions and 2-AG levels in the mouse liver.

Discussion

In this study, we demonstrated that activation of CB1R in hepatocytes promoted the expression of DAGLα and DAGLβ and increased liver 2-AG levels through ERRγ-mediated DAGLα and DAGLβ gene promoter activation leading to 2-AG production in hepatocytes. These results were supported by treatment with GSK5182, a selective ERRγ inverse agonist, which attenuated the alcohol feeding-induced upregulation of DAGLα and DAGLβ and 2-AG levels in the liver.

2-AG is synthesized by DAGLα and DAGLβ in the liver and brain (Bisogno et al. 2003; Gao et al. 2010). Alcohol feeding induces 2-AG production in hepatic stellate cells by upregulating DAGLβ expression (Jeong et al. 2008). Increased 2-AG levels in the liver activate CB1R signaling in hepatocytes, thereby activating the expression of lipogenic genes such as FAS and sterol regulatory element-binding protein-1c (Jeong et al. 2008). Moreover, previous study suggests that alcohol consumption increased hepatic glutamate release and mGluR5 (metabotropic glutamate receptor-5) stimulated 2-AG production via induction of DAGLβ gene expression in hepatic stellate cells (Choi et al. 2019). The present data suggested that CB1R activation increases ERRγ-mediated DAGLα and DAGLβ gene expressions and 2-AG levels in human hepatoma cells (HepG2) and mouse immortalized hepatocytes (AML12). Moreover, chronic alcohol feeding-induced DAGLα and β gene expressions and 2-AG levels were significantly attenuated in hepatocyte-specific CB1R knockout and GSK5182-treated mice. These data suggest an autocrine regulatory loop in which CB1-mediated ERRγ activation modulates DAGLα and β gene transcription in hepatocytes, leading to increased 2-AG levels in the liver. This indicates that in addition to hepatic stellate cells, hepatocytes are involved in alcohol exposure-induced 2-AG synthesis.

ERRγ binds to estrogen response elements as a dimer or to the half-site core sequence (TNAAGGTCA) as a monomer (Giguere et al. 1988; Luo et al. 2003; Razzaque et al. 2004). We previously showed that ERRγ directly binds to various gene promoters and regulates target gene promoter activity. We identified binding sites for ERRγ in the DAGLα and β promoters. The production of DAGLα- and DAGLβ-deficient mice was recently reported (Gao et al. 2010; Tanimura et al. 2010). Moreover, DAGL activity can be regulated by a synthetic agonist (Bisogno et al. 2006, 2009), although the DAGL synthetic agonist also inhibits other serine hydrolases (Hoover et al. 2008). In this study, GSK5182 decreased DAGLα and β promoter activity, inhibited DAGLα and β

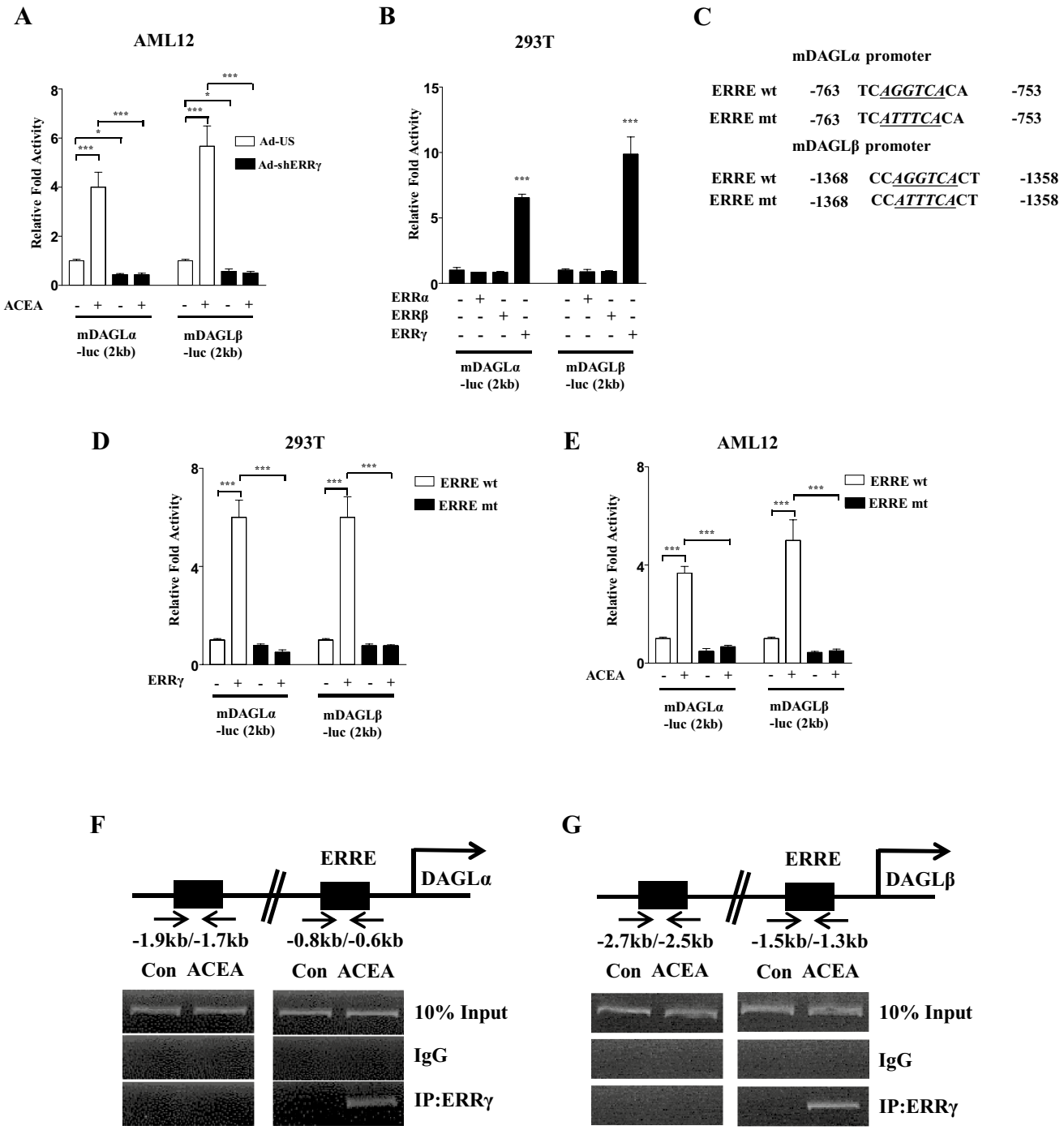


Fig. 3 ERRγ activates mouse DAGLα and β gene promoter activity. **a** AML12 cells were transfected with mDAGLα-Luc or mDAGLβ-Luc, and then stimulated with ACEA for 3 h. **b** 293T cells were transfected with mDAGLα-Luc or mDAGLβ-Luc, along with expression vectors for ERRα, ERRβ, or ERRγ. **c** The alignment of potential ERRE sequences in mouse DAGLα and β promoters is shown. **d** 293T cells were co-transfected with vectors expressing WT or ERRE mutant DAGLα and DAGLβ promoter luciferase constructs and ERR overexpression plasmids. **e** AML12 cells were transfected with WT or ERRE mutant DAGLα and DAGLβ promoter luciferase constructs and stimulated with ACEA for 3 h. **f**, **g** ChIP assay, showing binding of ERR to DAGLα and DAGLβ gene promoters. AML12 cells were treated with ACEA and soluble chromatin was immunoprecipitated with an ERR antibody. Purified DNA samples were used for PCR with primers that bind to the ERREs in DAGLα (−0.8 to −0.6 kb) and DAGLβ (−1.5 to −1.3 kb) gene promoters. All cell culture experiments were performed as three independent replicates. Data represent mean ± SEM. Data in **a**, **d**, **e** were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test, and data in **b** were analyzed by two-tailed Student's *t* test as $*p<0.05$; $***p<0.001$

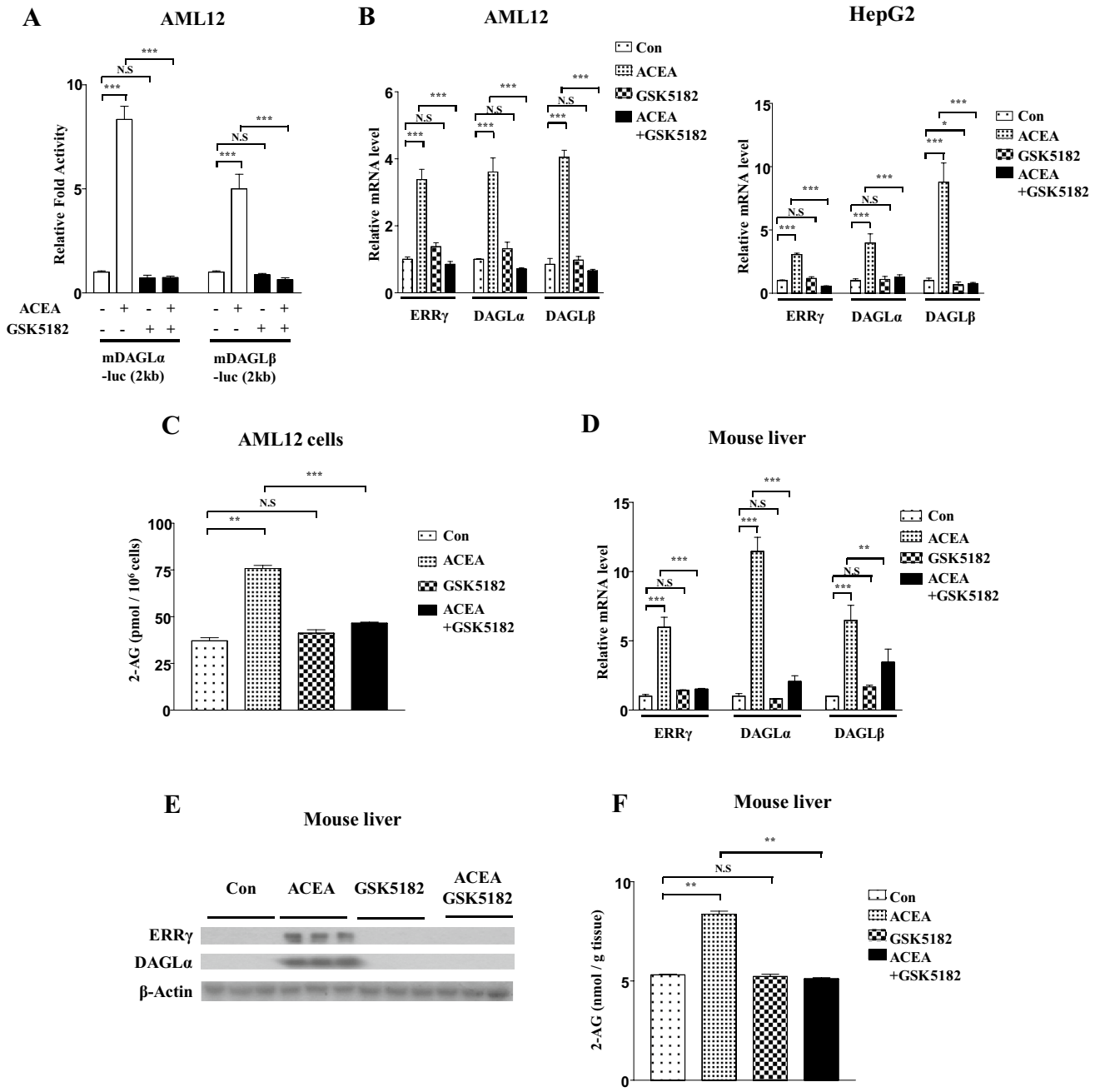


Fig. 4 GSK5182 inhibits ACEA-mediated induction of DAGLα and β gene expressions and 2-AG levels. **a** AML12 cells were transfected with mDAGLα-Luc or mDAGLβ–Luc, and stimulated with ACEA for 3 h in the presence or absence of GSK5182. **b** Q-PCR analysis of total RNAs isolated from AML12 and HepG2 cells treated with ACEA in the presence or absence of GSK5182. **c** Measurement of 2-AG levels from AML12 cells treated with ACEA in the presence or absence of GSK5182. Q-PCR analysis of total RNAs (**d**, $n=4$ per group), western blot analysis of total proteins (**e**, $n=3$ per group) and measurement of 2-AG levels (**f**, $n=4$ per group) from livers of mice treated with ACEA, with or without GSK5182. All cell culture experiments were performed as three independent replicates. Data represent mean ± SEM. All data were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test as $*p<0.05$; $**p<0.01$; $***p<0.001$; not significant (N.S)

gene expressions in response to ACEA under ethanol-fed conditions, and decreased 2-AG levels in alcohol-treated mice. Taken together, these results suggest that GSK5182 improves liver disease caused by ethanol feeding by regulating DAGLα and DAGLβ gene expressions at the transcriptional level and decreasing 2-AG levels.

The results of the present study indicate that CB1R-induced ERRγ regulates DAGLα and β mRNA and promoter

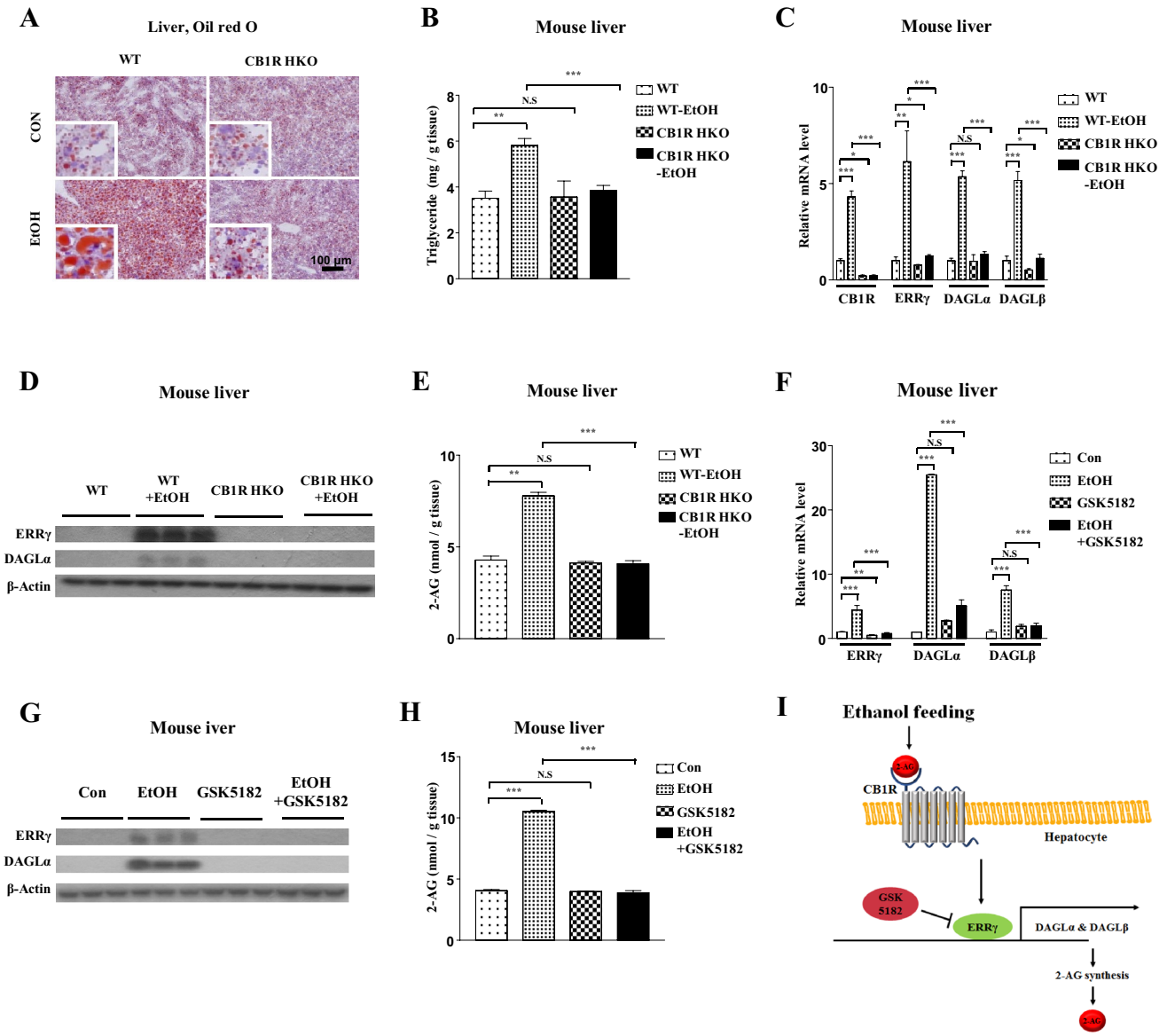


Fig. 5 Hepatocyte-specific CB1R knockout or GSK5182 treatment decreases chronic alcohol-feeding-induced DAGLα and DAGLβ gene expressions, and 2-AG levels. **a** Representative images of oil-red-O stained liver sections of control or ethanol fed WT and CB1R HKO mice. **b** Measuring triglyceride levels from control or ethanol fed WT and CB1R HKO mice livers ($n=6$ per group). Q-PCR analysis of total RNAs (**c**, $n=6$ per group), western blot analysis of total proteins (**d**, $n=3$ per group) and measurement of 2-AG levels (**e**, $n=6$ per group) from livers of control or ethanol fed WT and CB1R HKO mice. Q-PCR analysis of total RNAs (**f**, $n=5$ per group), western blot analysis of total proteins (**g**, $n=3$ per group) and measurement of 2-AG levels (**h**, $n=5$ per group) from livers of mice fed with ethanol with or without GSK5182. **i** Schematic diagram of ERRγ-mediated DAGLα and DAGLβ gene expression, which leads to and increase in 2-AG synthesis. Data represent mean ± SEM. All data were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test as $*p<0.05$; $**p<0.01$; $***p<0.001$; not significant (N.S)

activity, and GSK5182 inhibits hepatic CB1R-induced DAGLα and β gene expressions and 2-AG levels in hepatocytes (Fig. 5i). Inhibition of chronic alcohol-mediated liver damage and fatty liver by the ERRγ-specific inverse agonist GSK5182 may provide an attractive therapeutic strategy for the treatment of alcoholic liver disease.

Acknowledgements This research was supported by the National Creative Research Initiatives Grant (20110018305) and Basic Science Research Program (NRF-2015R1C1A1A01051513) through the National Research Foundation of Korea (NRF) funded by the Korean government (Ministry of Science and ICT). This work was also supported by the KRIBB Research Initiative Program of the Republic of Korea. C.S.C. was supported by Korea Mouse Phenotyping Project

(NRF-2014M3A9D5A01073886) of the Ministry of Science, ICT and Future Planning through the National Research Foundation. This research was supported by a Grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HI16C1501). This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (Grant number: NRF-2017R1A2B3006406) and the Cooperative Research Program for Agriculture Science and Technology Development (No. PJ01280701) funded by Rural Development Administration, Republic of Korea.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest to declare.

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

References

Arteel GE (2003) Oxidants and antioxidants in alcohol-induced liver disease. Gastroenterology 124:778–790. https://doi.org/10.1053/gast.2003.50087

Bisogno T et al (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163:463–468. https://doi.org/10.1083/jcb.200305129

Bisogno T et al (2006) Development of the first potent and specific inhibitors of endocannabinoid biosynthesis. Biochem Biophys Acta 1761:205–212. https://doi.org/10.1016/j.bbalip.2005.12.009

Bisogno T et al (2009) Synthesis and pharmacological activity of a potent inhibitor of the biosynthesis of the endocannabinoid 2-arachidonoylglycerol. ChemMedChem 4:946–950. https://doi.org/10.1002/cmdc.200800442

Cederbaum AI, Lu Y, Wu D (2009) Role of oxidative stress in alcohol-induced liver injury. Arch Toxicol 83:519–548. https://doi.org/10.1007/s00204-009-0432-0

Chao EY et al (2006) Structure-guided synthesis of tamoxifen analogs with improved selectivity for the orphan ERRgamma. Bioorg Med Chem Lett 16:821–824. https://doi.org/10.1016/j.bmcl.2005.11.030

Choi WM et al (2019) Glutamate signaling in hepatic stellate cells drives alcoholic steatosis. Cell Metab 30(877–889):e877. https://doi.org/10.1016/j.cmet.2019.08.001

Dey A, Cederbaum AI (2006) Alcohol and oxidative liver injury. Hepatology (Baltimore, Md) 43:S63–74. https://doi.org/10.1002/hep.20957

Gao B, Bataller R (2011) Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology 141:1572–1585. https://doi.org/10.1053/j.gastro.2011.09.002

Gao Y et al (2010) Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. J Neurosci 30:2017–2024. https://doi.org/10.1523/jneurosci.5693-09.2010

Garcia-Villafranca J, Guillen A, Castro J (2008) Ethanol consumption impairs regulation of fatty acid metabolism by decreasing the activity of AMP-activated protein kinase in rat liver. Biochimie 90:460–466. https://doi.org/10.1016/j.biochi.2007.09.019

Giguere V (2008) Transcriptional control of energy homeostasis by the estrogen-related receptors. Endocr Rev 29:677–696. https://doi.org/10.1210/er.2008-0017

Giguere V, Yang N, Segui P, Evans RM (1988) Identification of a new class of steroid hormone receptors. Nature 331:91–94. https://doi.org/10.1038/331091a0

Gonzalez FJ (2005) Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. Mutat Res 569:101–110. https://doi.org/10.1016/j.mrfmmm.2004.04.021

Gonzalez-Mariscal I et al (2018) Absence of cannabinoid 1 receptor in beta cells protects against high-fat/high-sugar diet-induced beta cell dysfunction and inflammation in murine islets. Diabetologia 61:1470–1483. https://doi.org/10.1007/s00125-018-4576-4

Hentschke M, Susens U, Borgmeyer U (2002) PGC-1 and PERC, coactivators of the estrogen receptor-related receptor gamma. Biochem Biophys Res Commun 299:872–879

Herzog B, Hallberg M, Seth A, Woods A, White R, Parker MG (2007) The nuclear receptor cofactor, receptor-interacting protein 140, is required for the regulation of hepatic lipid and glucose metabolism by liver X receptor. Mol Endocrinol (Baltimore, Md) 21:2687–2697. https://doi.org/10.1210/me.2007-0213

Hong H, Yang L, Stallcup MR (1999) Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 274:22618–22626

Hoover HS, Blankman JL, Niessen S, Cravatt BF (2008) Selectivity of inhibitors of endocannabinoid biosynthesis evaluated by activity-based protein profiling. Bioorg Med Chem Lett 18:5838–5841. https://doi.org/10.1016/j.bmcl.2008.06.091

Huss JM, Kopp RP, Kelly DP (2002) Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. J Biol Chem 277:40265–40274. https://doi.org/10.1074/jbc.M206324200

Jeong WI et al (2008) Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. Cell Metab 7:227–235. https://doi.org/10.1016/j.cmet.2007.12.007

Kim DK et al (2011) Estrogen-related receptor gamma (ERRgamma) is a novel transcriptional regulator of phosphatidic acid phosphatase, LIPIN1, and inhibits hepatic insulin signaling. J Biol Chem 286:38035–38042. https://doi.org/10.1074/jbc.M111.250613

Kim DK et al (2012) Orphan nuclear receptor estrogen-related receptor gamma (ERRgamma) is key regulator of hepatic gluconeogenesis. J Biol Chem 287:21628–21639. https://doi.org/10.1074/jbc.M111.315168

Kim DK et al (2013) Estrogen-related receptor gamma controls hepatic CB1 receptor-mediated CYP2E1 expression and oxidative liver injury by alcohol. Gut 62:1044–1054. https://doi.org/10.1136/gutjnl-2012-303347

Kim DK et al (2014a) Inverse agonist of estrogen-related receptor gamma controls Salmonella typhimurium infection by modulating host iron homeostasis. Nat Med 20:419–424. https://doi.org/10.1038/nm.3483

Kim DK et al (2014b) PKB/Akt phosphorylation of ERRgamma contributes to insulin-mediated inhibition of hepatic gluconeogenesis. Diabetologia 57:2576–2585. https://doi.org/10.1007/s00125-014-3366-x

Lee YS et al (2008) Orphan nuclear receptor SHP interacts with and represses hepatocyte nuclear factor-6 (HNF-6) transactivation. Biochem J 413:559–569. https://doi.org/10.1042/BJ20071637

Lee JH et al (2012) Hypoxia induces PDK4 gene expression through induction of the orphan nuclear receptor ERRgamma. PLoS ONE 7:e46324. https://doi.org/10.1371/journal.pone.0046324

Lieber CS, Schmid R (1961) The effect of ethanol on fatty acid metabolism; stimulation of hepatic fatty acid synthesis in vitro. J Clin Invest 40:394–399. https://doi.org/10.1172/jci104266

Lieber CS, Spritz N, DeCarli LM (1966) Role of dietary, adipose, and endogenously synthesized fatty acids in the pathogenesis of the alcoholic fatty liver. J Clin Investig 45:51–62. https://doi.org/10.1172/jci105323

Luo J, Sladek R, Carrier J, Bader JA, Richard D, Giguere V (2003) Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor alpha. Mol Cell Biol 23:7947–7956

Martin GG et al (2016) Female mice are resistant to Fabp1 gene ablation-induced alterations in brain endocannabinoid levels. Lipids 51:1007–1020. https://doi.org/10.1007/s11745-016-4175-4

Razzaque MA, Masuda N, Maeda Y, Endo Y, Tsukamoto T, Osumi T (2004) Estrogen receptor-related receptor gamma has an exceptionally broad specificity of DNA sequence recognition. Gene 340:275–282. https://doi.org/10.1016/j.gene.2004.07.010

Ryu D et al (2009) TORC2 regulates hepatic insulin signaling via a mammalian phosphatidic acid phosphatase, LIPIN1. Cell Metab 9:240–251. https://doi.org/10.1016/j.cmet.2009.01.007

Sanyal S, Kim JY, Kim HJ, Takeda J, Lee YK, Moore DD, Choi HS (2002) Differential regulation of the orphan nuclear receptor small heterodimer partner (SHP) gene promoter by orphan nuclear receptor ERR isoforms. J Biol Chem 277:1739–1748. https://doi.org/10.1074/jbc.M106140200

Sugiura T, Kobayashi Y, Oka S, Waku K (2002) Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. Prostagland Leukot Essent Fatty Acids 66:173–192. https://doi.org/10.1054/plef.2001.0356

Tanimura A et al (2010) The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. Neuron 65:320–327. https://doi.org/10.1016/j.neuron.2010.01.021

Ueda N, Tsuboi K, Uyama T, Ohnishi T (2011) Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. BioFactors (Oxford, England) 37:1–7. https://doi.org/10.1002/biof.131

Xie YB et al (2009) Transcriptional corepressor SMILE recruits SIRT1 to inhibit nuclear receptor estrogen receptor-related receptor gamma transactivation. J Biol Chem 284:28762–28774. https://doi.org/10.1074/jbc.M109.034165

You M, Fischer M, Deeg MA, Crabb DW (2002) Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). J Biol Chem 277:29342–29347. https://doi.org/10.1074/jbc.M202411200

You M, Matsumoto M, Pacold CM, Cho WK, Crabb DW (2004) The role of AMP-activated protein kinase in the action of ethanol in the liver. Gastroenterology 127:1798–1808

Zhang Y et al (2015) Orphan nuclear receptor oestrogen-related receptor gamma (ERRgamma) plays a key role in hepatic cannabinoid receptor type 1-mediated induction of CYP7A1 gene expression. Biochem J 470:181–193. https://doi.org/10.1042/bj20141494

Affiliations

Yoon Seok Jung[1] · Yong-Hoon Kim[2,3] · Kamalakannan Radhakrishnan[1] · Jina kim[4] · Don-Kyu Kim[1] · Ji-Hyeok Lee[5] · Hyunhee Oh[5] · In-Kyu Lee[6,7] · Wook Kim[8] · Sung Jin Cho[4,7] · Cheol Soo Choi[5,9] · Steven Dooley[10] · Josephine M. Egan[11] · Chul-Ho Lee[2,3] · Hueng-Sik Choi[1]

1 National Creative Research Initiatives Center for Nuclear Receptor Signals and Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Kwangju 61186, Republic of Korea

2 Laboratory Animal Resource Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Taejon 34141, Republic of Korea

3 Department of Functional Genomics, KRIBB School of Bioscience, Korea University of Science and Technology (UST), Taejon 34113, Republic of Korea

4 Daegu-Gyeongbuk Medical Innovation Foundation, New Drug Development Center, Taegu 41061, Republic of Korea

5 Lee Gil Ya Cancer and Diabetes Institute, Korea Mouse Metabolic Phenotyping Center, Gachon University, Inchon 21999, Republic of Korea

6 Department of Internal Medicine, School of Medicine, Kyungpook National University, Kyungpook National University Hospital, Taegu, Republic of Korea

7 Leading-Edge Research Center for Drug Discovery and Development for Diabetes and Metabolic Disease, Kyungpook National University Hospital, Taegu 41404, Republic of Korea

8 Department of Molecular Science and Technology, Ajou University, Suwon, Republic of Korea

9 Endocrinology, Internal Medicine, Gachon University Gil Medical Center, Inchon 21565, Republic of Korea

10 Department of Medicine II, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

11 Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA