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Estrogen-related receptor γ controls hepatic CB₁ receptor-mediated CYP2E1 expression and oxidative liver injury by alcohol

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Abstract

Background—The hepatic endocannabinoid system and cytochrome P450 2E1 (CYP2E1), a key enzyme causing alcohol-induced reactive oxygen species (ROS) generation, are major contributors to the pathogenesis of alcoholic liver disease. The nuclear hormone receptor oestrogen-related receptor (ERR) is a constitutively active transcriptional activator regulating gene expression.

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Competing interests None.

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Objective—To investigate the role of ERR α in the alcohol-mediated regulation of CYP2E1 and to examine the possibility to control alcohol-mediated oxidative stress and liver injury through an ERR α inverse agonist.

Design—For chronic alcoholic hepatosteatosis study, C57BL/6J wild-type and CB1 $^{-/-}$ mice were administered alcohol for 4 weeks. GSK5182 and chlormethiazole (CMZ) were given by oral gavage for the last 2 weeks of alcohol feeding. Gene expression profiles and biochemical assays were performed using the liver or blood of mice.

Results—Hepatic ERR α gene expression induced by alcohol-mediated activation of CB $_1$ receptor results in induction of CYP2E1, while liver-specific ablation of ERR α gene expression blocks alcohol-induced expression of CYP2E1 in mouse liver. An ERR α inverse agonist significantly ameliorates chronic alcohol-induced liver injury in mice through inhibition of CYP2E1-mediated generation of ROS, while inhibition of CYP2E1 by CMZ abrogates the beneficial effects of the inverse agonist. Finally, chronic alcohol-mediated ERR α and CYP2E1 gene expression, ROS generation and liver injury in normal mice were nearly abolished in CB1 $^{-/-}$ mice.

Conclusions—ERR α , as a previously unrecognised transcriptional regulator of hepatic CB $_1$ receptor, controls alcohol-induced oxidative stress and liver injury through CYP2E1 induction, and its inverse agonist could ameliorate oxidative liver injury due to chronic alcohol exposure.

INTRODUCTION

Alcoholic liver disease (ALD) caused by liver damage due to alcohol abuse is a major risk factor of morbidity and mortality worldwide.¹ Enhanced oxidative stress by the production of a variety of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide (H $_2$ O $_2$), lipid peroxides and peroxynitrite, is a key factor in the pathogenesis of ALD.^{2–4} Alcohol is predominantly oxidised to acetaldehyde via two well-characterised pathways in liver, alcohol dehydrogenase and the cytochrome P450-dependent microsomal ethanol oxidising system (MEOS). It is reported that MEOS activity plays a pivotal role in the increased capacity to oxidise alcohol (metabolic tolerance) found after chronic alcohol exposure. Cytochrome P450 2E1 (CYP2E1), a key enzyme of MEOS, has been shown to be a major contributor to alcohol-induced ROS and liver injury.^{3,56}

It has been reported that the endocannabinoid system, which consists of two G protein-coupled CB receptors, CB $_1$ and CB $_2$ receptors, is part of a complex lipid signalling network and that the two well-characterised endogenous cannabinoids are anandamide and 2-arachidonyl glycerol (2-AG).⁷ The CB $_1$ receptors are expressed in the brain and in various peripheral tissues, such as the heart, vascular tissues and liver, whereas the CB $_2$ receptors are expressed almost exclusively in the immune and haematopoietic cells. It has been demonstrated that the primary route of 2-AG synthesis is through hydrolysis of diacylglycerol (DAG) by diacylglycerol lipases (DAGL α and DAGL β), which contribute to the regulation of steady-state levels of 2-AG in brain and liver.⁸⁹ In addition, it has also been reported that 2-AG induction is regulated by alcohol-mediated DAGL α in stellate cells of liver, suggesting a paracrine mechanism by which hepatic stellate cell-derived 2-AG activates the CB $_1$ receptor on adjacent hepatocytes.¹⁰ Physiological studies have established that the hepatic endocannabinoid system is associated with the regulation of hepatic haemodynamics, fibrogenesis and lipid metabolism, and contributes to the pathogenesis of various liver diseases including cirrhosis, non-alcoholic fatty liver disease, alcoholic fatty liver and ischaemia–reperfusion injury.¹¹

Estrogen-related receptors (ERRs) belong to the NR3B subfamily which consists of three members: ERR α , ERR β and ERR γ . Crystallographic studies indicate that the ERRs are constitutively

active without a natural ligand, while several synthetic ligands either stimulate or repress the activity of the ERRs by promoting or disrupting ERR–coactivator interactions.¹² Among them, it has been reported that GSK5182, a 4-hydroxy tamoxifen analogue, is a selective inverse agonist of ERR α relative to other nuclear hormone receptors, and has antidiabetic effects through inhibition of hepatic gluconeogenesis in a PGC-1 α -dependent manner.¹³¹⁴ ERR isoforms are primarily expressed in heart, brain, kidney, pancreas and liver.¹⁵¹⁶ ERR α regulates mitochondrial programmes involved in oxidative phosphorylation and a nuclear-encoded mitochondrial genetic network that coordinates the postnatal metabolic transition in the heart.¹⁷¹⁸ On the other hand, it has been shown that hepatic ERR α is associated with the regulation of hepatic gluconeogenesis contributing to diabetes and is also involved in impaired insulin signalling through DAG-mediated protein kinase C α activation.¹⁴¹⁹ However, the function of hepatic ERR α in the pathogenesis of ALD remains largely unknown.

In the present study, we demonstrated that the orphan nuclear receptor ERR α controls alcohol-induced oxidative stress causing liver injury through induction of CYP2E1. Hepatic ERR α gene expression is induced by alcohol-mediated activation of CB $_1$ receptor signalling, which is responsible for the induction of the CYP2E1 gene. Hepatic ERR α expression led to the induction of CYP2E1, while ablation of hepatic ERR α gene expression blocked alcohol-induced expression of CYP2E1 in mice. An inverse agonist of ERR α ameliorated chronic alcohol-induced liver injury through inhibition of CYP2E1-mediated ROS generation in vivo. Control of alcohol-mediated oxidative stress production by an ERR α -specific inverse agonist could be a novel and alternative therapeutic approach for the treatment of ALD.

MATERIALS AND METHODS

Animal experiments

Male 8-week old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used for this study. CB $_1$ receptor knockout mice (CB $_1^{-/-}$) were kindly provided from Dr George Kunos at the National Institute on Alcohol Abuse and Alcoholism/NIH as described previously,²⁰²¹ and male 8-week old CB $_1^{-/-}$ mice were used in this chronic alcohol study. To identify the effect of ERR α or CB $_1$ receptor in an acute alcoholic liver injury model, normal and recombinant shERR α or shCB $_1$ -adenovirus delivered (intravenous) mice were injected with alcohol (6 g/kg, oral). For the compound studies, alcohol administration (6 g/kg, oral) was performed in normal mice preinjected with arachidonyl-2-chloroethylamide (AECA) (10 mg/kg, intraperitoneal). For the chronic alcoholic hepatosteatosis model, four groups of five mice each were treated for 4 weeks: (a) alcohol-containing Lieber-DeCarli formulation based liquid (Dyets, Bethlehem, Pennsylvania, USA) diet (27.5% of total calories), (b) pair-fed control diet in which alcohol was replaced isocalorically with carbohydrate, (c) control diet supplemented with GSK5182 (40 mg/kg, oral) and (d) alcohol-containing diet supplemented with GSK5182. In the last two groups, GSK5182 was injected once-daily for the last 2 weeks of the study. For the chlormethiazole (CMZ) experiments, during the 4 weeks of feeding with alcohol (27.5% of total calories) liquid diet, CMZ (50 mg/kg, intraperitoneal, every other day)²² or GSK5182 (40 mg/kg, oral, once-daily) was injected for the last 2 weeks into mice. All mice were acclimatised to a 12 h light–dark cycle at 22 $\pm$ 2°C with free access to food and water in a specific pathogen-free facility. All animal experiments were approved and performed by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology.

Preparation of liver mitochondria, cytosol and microsomes

In order to prepare hepatic subcellular fractions, mitochondria, microsomes and cytosol were isolated as previously described with some modifications.²³ Briefly, immediately after

decapitation, the livers were homogenised in 2–5 volumes of 100 mM Tris-HCl (pH 7.4, 4°C) containing 100 mM KCl, 1 mM EDTA, 2 mM phenylmethyl sulphonyl fluoride and a protease inhibitor, using a homogeniser (IKA Labortechnik, Selayasia, Malaysia) and the homogenate was centrifuged at 1000×g for 15 min (4°C). The supernatant was centrifuged at 10 000×g for 15 min (4°C) and the pellet (containing mitochondria) was kept at –80°C. The 10 000×g supernatant was centrifuged at 100 000×g for 60 min (4°C), the pellet (microsomal fraction) was resuspended in 10 mM Tris-HCl (pH 7.4, 4°C) containing 1 mM EDTA and 20% glycerol and was stored at –80°C. The supernatant (cytosol) was also kept at –80°C. The protein concentrations were estimated using a bicinchoninic acid procedure (Pierce, Rockford, Illinois, USA) with bovine albumin solution as the standard.

Measurement of CYP2E1 enzyme activity

The assay of CYP2E1 activity was done using chlorzoxazone as described elsewhere with slight modifications.²⁴ The standard incubation mixture (final volume of 0.25 ml) contained liver microsomes (100 µg protein) and chlorzoxazone (100 µM) in 100 mM potassium phosphate buffer (pH 7.4). The reaction was started by adding an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate and 0.5 unit of glucose-6-phosphate dehydrogenase/ml), and after the sample was incubated at 37°C for 20 min, the reaction was stopped by adding 25 µl of 43% H₃PO₄ and 0.75 ml of CH₂Cl₂. Organic phases were evaporated under a nitrogen stream, and product formation was determined by high-performance liquid chromatography with a C8 column (4.6×250 mm, 5 µm). The elution was conducted with a mixture of 27% CH₃CN (v/v) in 0.5% aqueous H₃PO₄ (w/v) at a flow rate of 1.0 ml/min, and detection was by UV absorbance at 287 nm.

Measurement of hydrogen peroxide production

Reaction systems were prepared as describe above. Reactions were initiated by addition of the NADPH-generating system. The concentration of H₂O₂ was measured spectrophotometrically using PeroXOquant according to the manufacturer's instructions (PIERCE, Rockford, Illinois, USA).²⁵

TUNEL assay

Terminal transferase deoxyuridine triphosphate nick end labelling (TUNEL) assay was performed using sections of paraffin-embedded tissue samples according to the method of ApopTag plus peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, California, USA). For detection of apoptotic cells, fragmented DNA of apoptotic cells was deoxygenated by terminal deoxynucleotidyl transferase. The digoxigenin was labelled by anti-digoxigenin-peroxidase and visualised by 3, 3'-diaminobenzidine.

Statistical analysis

Data are expressed as means±SEM. Statistical analysis was performed using the two-tailed Student t test or one-way ANOVA. Differences were considered statistically significant at p<0.05.

Additional methods

Detailed methodology is described in the online supplementary Materials and methods section.

RESULTS

Hepatic *ERR γ* and *CYP2E1* gene expression is regulated by alcohol-mediated activation of CB₁ receptor signalling

In an effort to explore the function of orphan nuclear receptor *ERR* in alcoholic liver injury, we first examined *ERR* gene expression in the liver of mice administered alcohol. Interestingly, expression of hepatic *ERR γ* , but not *ERR α* , was significantly induced by acute alcohol feeding (figure 1A). Expression of *CYP2E1* was also upregulated under this condition. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, markers of liver injury, were higher in alcohol-exposed mice (see online supplementary figure S1A). Consistent with the regulation of hepatic endocannabinoid system by alcohol,¹⁰ 2-AG levels were elevated in alcohol-fed mice (figure 1B). In line with these changes, mRNA levels of diacylglycerol lipase (*Dagl*), a 2-AG biosynthetic enzyme,⁸ were selectively increased in liver of mice acutely exposed to alcohol (figure 1C).

To further examine the regulation of hepatic *ERR* and *CYP2E1* genes by alcohol-mediated induction of endocannabinoids, we analysed mRNA levels for *ERR* and *CYP2E1* in the liver of mice treated with 2-AG ether in a time-dependent manner. Surprisingly, mRNA levels of *ERR γ* were markedly enhanced only 3 h after 2-AG ether treatment and remained elevated until 24 h, while the induction of CB₁ receptor mRNA was increased within 1 h-treatment of 2-AG ether and gradually induced until 12 h (see online supplementary figure S1B). However, *ERR α* was not changed until 24 h. On the other hand, *CYP2E1* mRNA was only modestly induced between 1 and 3 h after 2-AG ether treatment, and strongly induced after 6 h, indicating that the endocannabinoid receptor-mediated induction of *ERR* expression precedes that of *CYP2E1* gene expression in liver of mice. Consistent with the change of *ERR* and *CYP2E1* mRNAs, protein levels were highly induced in the livers of mice treated with 2-AG ether (figure 1D). Plasma AST and ALT levels were also elevated in 2-AG ether-treated mice compared with control mice (see online supplementary figure S1C). Consistent with these results, administration of ACEA, a selective CB₁ agonist, strongly induced hepatic *ERR* and *CYP2E1* expression in mice (figure 1E). To further confirm the induction of *ERR* and *CYP2E1* via endocannabinoid signalling, we employed a selective antagonist of CB₁, AM251. Administration of AM251 to mice significantly attenuated 2-AG ether-mediated hepatic *ERR* and *CYP2E1* gene induction (see online supplementary figure S1D). In addition, modelling hepatic CB₁ receptor deficiency using adenoviral-mediated overexpression of shCB₁ led to a reduction of the basal and alcohol-induced hepatic *ERR* and *CYP2E1* expression in mice (figure 1F), suggesting clearly that the CB₁ receptor is required for alcohol-mediated regulation of *ERR* and *CYP2E1*.

To exclude the potential secondary action of 2-AG on the induction of *ERR* and *CYP2E1* gene expression, we also confirmed the 2-AG effect in rat primary hepatocytes. Similar with the result in liver of mice, *ERR* and *CYP2E1* mRNA levels were significantly increased by 2-AG ether treatment (figure 1G). The CB₁ receptor is linked to the activation of c-Jun N-terminal kinase (JNK),^{26,27} and we confirmed the involvement of JNK signalling in regulation of *ERR* expression by 2-AG in rat primary hepatocytes. As expected, 2-AG ether-induced mRNA levels of *ERR* and *CYP2E1* were significantly decreased by treatment with SP600125, a specific JNK inhibitor (see online supplementary figure S2A). We also found that 2-AG ether-mediated activation of the *ERR* promoter was almost blocked by treatment of AM251 and co-transfection with the dominant negative form of c-Jun (c-Jun DN) (see online supplementary figure S2B). We identified a conserved AP1 consensus sequence in both human and mouse *ERR* promoters and confirmed its functional significance using site-specific mutagenesis analysis of this site using transient transfection assays (figure 1H, see online supplementary figure S2C). In addition, chromatin immunoprecipitation (ChIP) showed that 2-AG ether-mediated occupancy of c-Jun on *ERR*

promoter was completely blocked by treatment of SP600125 (figure 1I). Taken together, these results indicate that hepatic ERR and CYP2E1 expression is regulated by alcohol-mediated activation of CB₁ receptor at the transcriptional level.

ERR γ is a transcriptional regulator of hepatic CYP2E1 gene expression in vivo

To test the potential role of ERR as a direct inducer of hepatic CYP2E1 gene expression, we used adenoviral overexpression and knockdown of ERR in C57BL/6J mice. As expected, Ad-ERR elicited a marked increase in hepatic CYP2E1 mRNA and protein levels (figure 2A,B). Expression of pyruvate dehydrogenase kinase 4, a known ERR target gene, was also higher in Ad-ERR infected mouse liver compared with control. Decreasing endogenous ERR expression in liver of mice using Ad-shERR modestly decreased basal CYP2E1 expression and strongly decreased the induction of CYP2E1 mRNA and protein in response to acute alcohol administration (figure 2C,D). To examine the cell-autonomous effect of ERR overexpression or knockdown on CYP2E1 expression, we carried out adenoviral infection of ERR and treatment of 2-AG ether in rat primary hepatocytes and AML12 cells, respectively. Indeed, ERR and 2-AG ether significantly induced gene expression of CYP2E1 in both hepatocytes (figure 2E, see online supplementary figure S3A). Interestingly, Ad-ERR had no effect on CYP2E1 expression in rat primary hepatocytes (see online supplementary figure S3B). Conversely, adenovirus-mediated knockdown of ERR in rat primary hepatocytes largely attenuated the 2-AG ether-mediated induction of CYP2E1 expression (see online supplementary figure S3C), confirming that the effect of adenoviral overexpression or knockdown of ERR in mice could be reproduced in primary hepatocytes.

To explore the molecular mechanisms underlying the regulation of CYP2E1 gene transcription by ERR, we first tested CYP2E1 promoter transactivation by ERR subfamily members in HepG2 cells using transient transfection assays. ERR specifically increased CYP2E1 promoter activity in a dose-dependent manner, and ERR or ERR did not (see online supplementary figure S3D). Based on the induction of CYP2E1 promoter activity by ERR, the change of CYP2E1 mRNA and protein expression by 2-AG or ERR may reflect either an increased transcription rate of the CYP2E1 gene or a stabilisation of the mRNA. To examine whether CYP2E1 expression is regulated at transcriptional or post-transcriptional level, we employed treatment of Actinomycin D (Act D), a transcriptional inhibitor, in the presence of 2-AG ether or ERR. The mRNA levels of CYP2E1 were significantly increased by treatment of 2-AG ether or overexpression of ERR, which corresponds to the protein levels of CYP2E1 (figure 2F, see online supplementary figure S3E), whereas 2-AG or ERR-mediated increase of CYP2E1 mRNA and protein levels was almost entirely blocked by treatment of Act D. We identified a conserved ERR responsive element in both human and mouse CYP2E1 promoters (see online supplementary figure S3F) and demonstrated that it is required for transactivation of the CYP2E1 promoter by ERR (figure 2G). Finally, we confirmed the direct regulation of CYP2E1 transcription by ERR using ChIP assay. ERR was strongly recruited to the ERR responsive element region of the CYP2E1 promoter in the presence of 2-AG ether, which was significantly decreased by SP600125 treatment (figure 2H). We conclude that ERR directly induces gene transcription of CYP2E1.

GSK5182 ameliorates oxidative liver injury by chronic alcohol exposure in mice

Based on the molecular mechanism regarding regulation of CYP2E1 gene expression by ERR, we next tested if control of ERR transcriptional activity by its specific inverse agonist, GSK5182, could contribute to amelioration of alcoholic liver injury through inhibition of CYP2E1-induced oxidative stress. As expected, transactivation of the CYP2E1 promoter was markedly decreased by treatment of GSK5182 in a dose-dependent manner

(figure 3A). In addition, ERR γ -mediated induction of CYP2E1 protein was significantly decreased by GSK5182 treatment in rat primary hepatocytes and HepG2 cells, and this response was lost with the ERR γ Y326A mutant, which is not able to interact with GSK5182 (figure 3B,C). Consistent with the results in cultured cells, GSK5182 strongly inhibited both basal and alcohol-induced hepatic CYP2E1 mRNA and protein expression in liver of mice (figure 3D,E).

To examine the effect of GSK5182 on CYP2E1-mediated oxidative stress and liver injury in chronic alcohol-exposed C57BL/6J mice, alcohol was administered for 4 weeks and GSK5182 was given by oral gavage administration once-daily for the last 2 weeks of alcohol feeding. Chronic alcohol consumption significantly increased hepatic *Dagl1* and *Dagl1* mRNA levels (see online supplementary figure S4A) and ERR γ , CYP2E1 and CB $_1$ gene expression (figure 4A). The induction of CYP2E1 expression by alcohol was greatly decreased by GSK5182 treatment in both in basal and alcohol-induced conditions, but there was no effect on CB $_1$ or ERR γ expression (figure 4A, see online supplementary figure S4B). GSK5182 also significantly decreased both basal and chronic alcohol-induced CYP2E1 enzyme activity (figure 4B). In response to chronic alcohol consumption, hepatic CYP2E1 enhances ROS production, including H $_2$ O $_2$ and 4-hydroxynonenal (4-HNE), leading to mitochondrial damage, DNA modification and cell death.⁶ We assessed ROS levels by measuring H $_2$ O $_2$ production using hepatic microsomes and levels of hepatic 4-HNE using immunofluorescence. Treatment with the inverse agonist significantly reduced chronic alcohol-enhanced H $_2$ O $_2$ and 4-HNE production (figure 4C,D). On the other hand, the increase of mitochondrial apoptotic markers such as cytochrome C, Smac, endonuclease G and cleaved caspase 3 by chronic alcohol treatment was markedly reduced by GSK5182 treatment (figure 4E, see online supplementary figure S4C). TUNEL assay showed that the induction of apoptotic cell death upon chronic alcohol exposure was nearly eliminated by GSK5182 administration (figure 4F). Plasma AST and ALT levels indicated that GSK5182 markedly decreased chronic alcohol-mediated liver toxicity (figure 4G). Taken together, we conclude that the ERR γ inverse agonist ameliorates alcoholic liver damage via inhibition of CYP2E1-dependent ROS generation.

GSK5182 ameliorates alcoholic liver damage through inhibition of CYP2E1

To demonstrate the specific regulation of CYP2E1 by GSK5182 in alcoholic liver injury in mice, we used a CYP2E1 inhibitor (CMZ).^{28,29} Mice were administered alcohol for 4 weeks, and GSK5182 or CMZ was given for the last 2 weeks of alcohol feeding. Similar with previous reports,^{28,29} CMZ significantly decreased alcohol-mediated induction of CYP2E1 expression at the transcriptional level (figure 5A). As expected, alcohol-mediated production of 4-HNE and increased mitochondrial apoptotic markers were markedly decreased by either CMZ or GSK5182 treatment (figure 5B,C). Notably, the magnitude of their reductions by CMZ is almost similar to that by GSK5182, which was recapitulated in the amelioration of alcohol-mediated liver toxicity and apoptotic cell death by CMZ or GSK5182 (figure 5D,E). Interestingly, there was no further reduction in 4-HNE production, mitochondrial apoptotic markers, liver toxicity and apoptotic cell death by simultaneous treatment of CMZ and GSK5182. These results suggest that CYP2E1 mediates the effect of GSK5182 inhibition of alcohol-induced ROS generation.

ERR γ is a transcriptional mediator of CB $_1$ receptor in alcoholic liver injury

It has been reported that the hepatic endocannabinoid system is associated with the regulation of hepatic lipid metabolism and fibrogenesis, and contributes to the pathogenesis of various liver diseases including alcoholic fatty liver and cirrhosis.¹¹ However, the relevance of hepatic CYP2E1, ROS generation and CB $_1$ in the pathogenesis of these diseases remains unknown. Therefore, we next questioned whether hepatic CB $_1$ receptor

could mediate ROS-induced liver injury by alcohol because the activation of hepatic CB₁ receptor induces ERR gene expression leading to CYP2E1-mediated ROS generation. Wild-type and CB₁ receptor knockout (CB₁^{-/-}) mice were administered vehicle or alcohol for 4 weeks. Consistent with the results in liver of mice with ablated hepatic ERR gene expression (figure 1F), the induction of hepatic ERR and CYP2E1 protein as well as mRNA by chronic alcohol exposure in control mice was nearly abolished in CB₁^{-/-} mice (figure 6A,B). As expected, alcohol-mediated hepatic 4-HNE production was significantly attenuated in CB₁^{-/-} mice (figure 6C,D). In addition, the increase of cytochrome C, Smac, endonuclease G and cleaved caspase 3 by alcohol treatment in wild-type mice was markedly reduced in CB₁^{-/-} mice (figure 6E), which is further supported by the TUNEL assay showing the decreased apoptotic cell death in CB₁^{-/-} mice administered alcohol (figure 6F). Concurrently, plasma AST and ALT levels in CB₁^{-/-} mice were lower than those of wild-type mice when fed alcohol (figure 6G). Taken together, these results suggest that hepatic CB₁ receptor causes alcoholic liver injury through ERR-mediated induction of CYP2E1.

DISCUSSION

ALD is mainly caused by alcohol hepatotoxicity linked to its metabolism by means of the alcohol dehydrogenase and MEOS pathways and the resulting production of toxic acetaldehyde. It is well documented that enhanced oxidative stress by alcohol-mediated accumulation of ROS is a major factor in the pathogenesis of alcohol-induced liver disease.³ Indeed, many pathways have been suggested to contribute to the ability of alcohol to induce a state of oxidative stress leading to liver damage.³ Among them, abundant evidence indicates that cytochrome P450-dependent MEOS may play an important role in metabolic tolerance for chronic alcohol exposure and ROS generation.³⁰ Particularly, alcohol-mediated induction of CYP2E1 is known to be a major pathway of ROS generation and enhanced oxidative stress due to its high catalytic activity with alcohol.³⁰ However, considerable data have been collected to demonstrate that the regulation of CYP2E1 protein by alcohol is somewhat more complicated, being confirmed at the transcriptional, translational and post-transcriptional levels.³¹ In the present study, we found that hepatic ERR expression was induced by alcohol exposure in a CB₁ receptor-dependent manner and was responsible for induction of CYP2E1 in liver of mice (figure 6H). While overexpression of ERR in liver of mice led to induction of CYP2E1, alcohol-induced CYP2E1 expression was blunted by ablation of hepatic ERR expression. We also showed ERR-mediated transcriptional regulation of CYP2E1 gene by transient transfection assay using its promoter and by ChIP assay. Furthermore, hepatic CYP2E1 mRNA or its promoter activity was positively regulated by 2-AG ether treatment, which was blocked by knockdown of ERR or specific mutation of the ERR binding site on the CYP2E1 promoter. In addition, 2-AG or ERR-mediated increase of CYP2E1 mRNA and protein levels was almost entirely blocked by treatment of Act D, compared with those in the absence of Act D (figure 2F), indicating that CYP2E1 gene expression is regulated by ERR at the transcriptional level in a CB₁ receptor-dependent manner.

It has been reported that the primary route of 2-AG synthesis is through hydrolysis of DAG by DAGL and DAGL, which contribute to the regulation of steady-state levels of 2-AG in brain and liver.⁸⁹ In addition, it has been shown that 2-AG induction is regulated by alcohol-mediated DAGL in stellate cells of liver, suggesting a paracrine mechanism by which hepatic stellate cells-derived 2-AG activates the CB₁ receptor on adjacent hepatocytes.¹⁰ Similar with the report, we also found that DAGLs are significantly induced by chronic alcohol treatment. On the other hand, it has been reported that 2-AG binds with the same affinity to CB₁ and CB₂ and acts as a full agonist at both receptors,³² indicating that 2-AG is a non-selective CB receptor ligand. However, interestingly, the tissue distribution of CB receptors is somewhat different.¹¹³³ For example, CB₁ receptor is highly expressed in the

brain but also present in peripheral tissue, such as the heart, vascular tissues and liver, while CB₂ receptor is primarily expressed in immune and haematopoietic cells. In addition, CB₁ receptor in liver exhibits low-level expression in hepatocytes, stellate cells and hepatic vascular endothelial cells, while CB₂ receptor is undetectable in the normal liver but is induced in the embryonic state and pathological conditions such as non-alcoholic fatty liver disease, liver fibrosis, regenerating liver and hepatocellular carcinoma, suggesting that the different distribution of CB receptors could determine their role in ALDs. Recently, it has been reported that chronic alcohol treatment mediates an inflammatory response in Kupffer cells of the liver, which is inhibited by the activation of the CB₂ receptor by a selective CB₂ receptor agonist, JWH133, thereby reducing hepatocyte steatosis.³⁴ These findings suggest that the alcohol treatment could cause fat accumulation in liver and an inflammatory response in Kupffer cells, and alcohol-mediated 2-AG induction would selectively activate CB₁ receptors in Kupffer cells and hepatocytes. This notion is further supported, in part, by the findings that in patients infected with hepatitis C virus, daily cannabis treatment enhanced fibrosis progression instead of protecting patients against it and that 2-AG is the likely fibrogenic mediator because its hepatic level is preferentially increased by CCl₄ treatment in rodents, suggesting that endocannabinoids mediate a profibrotic effect, possibly through CB₁ receptors.^{35–37} Therefore, this could suggest an additional role of ERR in Kupffer cells, as well hepatocytes, in response to alcohol. Future studies will reveal the extent to which ERR could mediate the alcohol-induced inflammatory response through CB₂ receptors in Kupffer cells of liver.

It is reported that activation of the hepatic CB₁ receptor by endocannabinoids derived from stellate cells in response to alcohol is associated with alcoholic fatty liver.¹⁰ It is also known that activation of CB₁ receptor signalling by endocannabinoids is involved in the activation of mitogen-activated protein kinase (MAPK) including extracellular signal-regulated kinase, JNK and p38 MAPK, leading to cell death.²⁷ We showed that 2-AG-mediated induction of ERR mRNA and promoter activity was significantly decreased by a JNK inhibitor and dominant negative form of c-Jun, respectively, and it was also confirmed by ChIP assay, suggesting that ERR functions as a JNK-dependent downstream mediator of hepatic CB₁ receptor signalling. Interestingly, it has been reported that alcohol-mediated generation of ROS and reactive nitrogen species is a major activator of JNK that leads to liver cell death,^{38,39} suggesting that ERR acts as a cell signal amplifier of alcohol-mediated JNK activation.

Alcohol-exposed rodents and human alcoholics produce greater amounts of ROS from elevated CYP2E1 expression, and CYP2E1 induction by chronic and binge alcohol exposure is considered a major contributor to ALD.^{40–43} Indeed, several CYP2E1 inhibitors have been proposed as candidates for mini-mising alcohol-enhanced hepatotoxicity.³ On the other hand, it has been reported that the hepatic CB₁ receptor is associated with fatty liver, steatosis, dyslipidaemia by a high-fat diet or chronic alcohol feeding, and also contributes to diet-induced insulin resistance.^{11,44} However, to date, downstream effectors mediating hepatic CB₁ receptor signalling remain largely unknown and the therapeutic potential of hepatic CB₁ blockade is limited due to neuropsychiatric side effects. Our results demonstrate that ERR is a transcriptional mediator of hepatic CB₁ receptor, leading to the CYP2E1-induced oxidative stress and alcoholic liver injury, and its inverse agonist GSK5182 significantly inhibits chronic alcohol-mediated induction of CYP2E1 enzyme activity, ROS generation, and strongly ameliorates liver damage. Inhibition of alcohol-mediated oxidative stress by an ERR-specific inverse agonist may be a novel and attractive therapeutic approach for amelioration of alcoholic liver injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance of this study

What is already known on this subject?

Cytochrome P450 2E1 (CYP2E1), a key enzyme causing alcohol-induced reactive oxygen species generation and liver injury, is a major contributor in the pathogenesis of alcoholic liver disease (ALD).

Hepatic CB₁ is associated with the regulation of hepatic lipid metabolism and fibrogenesis, and contributes to the pathogenesis of alcoholic fatty liver and cirrhosis.

Hepatic oestrogen-related receptor (ERR) contributes to hepatic glucose production and impaired insulin signalling, causing type 2 diabetes.

What are the new findings?

Orphan nuclear receptor ERR is a previously unrecognised transcriptional regulator of hepatic CB₁ receptor, contributing to the pathogenesis of ALD.

ERR controls alcohol-induced oxidative stress and liver injury through induction of CYP2E1.

An ERR inverse agonist ameliorates chronic alcohol-induced liver injury through inhibition of CYP2E1-mediated oxidative stress.

How might it impact on clinical practice in the foreseeable future?

Suppression of alcohol-mediated oxidative stress and liver injury by an ERR-specific inverse agonist may be a novel and attractive therapeutic approach for the treatment of ALD.

Figure 1.

Alcohol feeding induces hepatic *ERR* and *CYP2E1* gene expression via hepatic *CB1* receptor signalling. (A–D) Male C57BL/6J mice (n=5 per group) were administered either vehicle or liquid alcohol (6 g/kg) for 12 h. (A) Q-PCR analysis of total RNAs isolated from mouse liver. (B) 2-Arachidonyl glycerol (2-AG) levels in liver. (C) Q-PCR analysis showing mRNA levels of *Daglα* and *Daglβ* in liver. (D) Western blot analysis (top) and graphical representation (bottom) showing *ERR* and *CYP2E1* in livers of mice (n=4 per group) administered 2-AG ether (5 mg/kg) for 12 h. (E) Male C57BL/6J mice (n=5 per group) were administered either vehicle or ACEA (10 mg/kg) for 24 h. Q-PCR analysis of total RNAs isolated from mouse liver. (F) Either Ad-US or Ad-sh*CB1* was injected via tail-vein of male C57BL/6J mice (n=5 per group). Mice were administered vehicle or alcohol for 24 h at day 5 after the injection. Q-PCR analysis showing mRNA levels of *ERR*, *CYP2E1* and *CB1* in liver of mice. (G) Q-PCR analysis showing *ERR*, *CYP2E1* and *CB1* expression in rat primary hepatocytes stimulated by 2-AG ether for 12 h. (H) AP-1-dependent activation of *ERR* promoter. Transient transfection was carried out in HepG2 cells along with the indicated plasmids. (I) ChIP assay showing the binding of c-Jun to *ERR* promoter. Non-transformed mouse liver (AML12) cells were treated with 2-AG ether (10 μM) and SP600125 (20 μM) for 12 h and soluble chromatin was immunoprecipitated with c-Jun antibody. Error bars show ± SEM *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student t test.

Figure 2.

ERR regulates hepatic CYP2E1 expression in liver of mice. (A, B) Ad-green fluorescent protein (GFP) or Ad-ERR were injected via tail-vein into male C57BL/6J mice (n=3–4 per group) and mice were sacrificed at day 7. (A) Q-PCR analysis showing mRNA levels of CYP2E1 and PDK4 in liver of mice. (B) Western blot analysis (top) and graphical representation (bottom) showing CYP2E1 expression in livers of mice. (C, D) Ad-US or Ad-shERR were injected via tail-vein of male C57BL/6J mice (n=5 per group). Mice were administered vehicle or alcohol (6 g/kg) for 24 h at day 5 after the injection. (C) Q-PCR analysis showing mRNA levels of CYP2E1 and ERR in liver of mice. (D) Western blot analysis (top) and graphical representation (bottom) showing CYP2E1 expression in livers of mice. (E) Q-PCR analysis showing mRNA levels of CYP2E1, CB₁ and PDK4 in rat primary hepatocytes infected with Ad-GFP or Ad-ERR. (F) Effect of ERR on CYP2E1 gene expression in the presence or absence of Actinomycin D (Act D). Act D (10 µg/ml) was added to AML12 cells infected with Ad-ERR. (G) ERR responsive element (ERRE)-dependent activation of mCYP2E1 promoter in HepG2 cells. (H) ChIP assay showing the binding of ERR to CYP2E1 promoter. AML12 cells were treated with 2-arachidonyl glycerol (2-AG) ether (10 µM) and SP600125 (20 µM) for 12 h. Error bars show ±SEM. *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student t test.

Figure 3.

Inverse agonist of ERR inhibits acute alcohol-mediated hepatic CYP2E1 expression in mice. (A) Effect of GSK5182 on *ERRγ*-induced CYP2E1 promoter activity. HepG2 cells were transfected with the indicated plasmids and treated with GSK5182 (10 μM) for 24 h. (B) *ERRγ*-specific inhibition of GSK5182 for CYP2E1 gene expression. Rat primary hepatocytes were infected with the indicated adenovirus and treated with GSK5182 (10 μM) for 24 h. (C) HepG2 cells were infected with the indicated adenovirus and treated with GSK5182 (10 μM) for 24 h. (D, E) Vehicle and GSK5182 (40 mg/kg once daily) were intraperitoneally injected in male C57BL/6J mice (n=5 per group) for 3 days. Alcohol (6 g/kg) was administered for 24 h at day 3 after once-daily injection of GSK5182. (D) Q-PCR analysis showing mRNA levels of *CYP2E1* and *ERRγ* in liver of mice. (E) Western blot analysis (top) and graphical representation (bottom) showing CYP2E1 expression in liver of mice. Error bars show ±SEM *p<0.05 by two-tailed Student t test.

Figure 4.

ERR inverse agonist ameliorates alcoholic liver injury. (A–G) Male C57BL/6J mice (n=5 per group) were fed an alcohol-containing diet for 4 weeks and GSK5182 (40 mg/kg once daily) was given by oral gavage for the final 2 weeks of alcohol feeding. (A) Q-PCR analysis showing mRNA levels of hepatic CYP2E1, CB₁ and ERR in mice. (B, C) Microsomes were isolated from liver of mice. The enzyme activity of CYP2E1 (B) and H₂O₂ production (C) were analysed. (D) Immunofluorescence showing 4-hydroxynonenal (4-HNE) in livers of mice (left). Quantitation of immunofluorescence showing 4-HNE (right). (E) Western blot analysis showing cytosolic cytochrome C, Smac, endo G and cleaved caspase 3 levels in livers of mice. (F) TUNEL assay showing apoptotic hepatocytes in liver of mice. (G) Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Error bars show $\pm$ SEM *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student t test. Different lowercase letters in (C) show statistical significance (p<0.05) by one-way ANOVA among groups.

Figure 5.

GSK5182 improves alcoholic liver injury through inhibition of CYP2E1. (A–E) Male C57BL/6J mice (n=5 per group) were fed an alcohol-containing diet for 4 weeks and GSK5182 (40 mg/kg, oral, once daily) or chlormethiazole (50 mg/kg, intraperitoneal, every other day) was given for the final 2 weeks of alcohol feeding. (A) Q-PCR analysis showing mRNA levels of hepatic *ERRγ* and *CYP2E1* in mice. (B) Immunofluorescence images showing 4-hydroxynonenal (4-HNE) in livers of mice (left). Quantitation of immunofluorescence showing 4-HNE (right). (C) Western blot analysis showing cytosolic cytochrome C, Smac, endo G and cleaved caspase 3 levels in livers of mice. (D) TUNEL assay showing apoptotic hepatocytes in liver of mice. (E) Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student t test. Different lowercase letters in (B) and (E) show statistical significance (p<0.05) by one-way ANOVA among groups.

Figure 6.

ERR is a downstream mediator of CB₁ receptor in CYP2E1-induced alcoholic liver injury. (A–G) Either vehicle or liquid alcohol was administered to wild-type and CB1^{-/-} mice (n=3–4) for 4 weeks. (A) Q-PCR analysis of total RNAs isolated from mouse livers. (B) Western blot analysis showing ERR and CYP2E1 in livers of mice. (C) Immunofluorescence showing 4-hydroxynonenal (4-HNE) in livers of mice. (D) Quantitation of immunofluorescence showing 4-HNE. (E) Western blot analysis showing cytosolic cytochrome C, Smac, endo G and cleaved caspase 3 levels in livers of mice. (F) TUNEL assay showing apoptotic hepatocytes in liver of mice. Blanked arrow indicates apoptotic cell. (G) Plasma aspartate aminotransferase and alanine aminotransferase levels. (H) A proposed model. Alcohol-mediated hepatic ERR expression enhances CYP2E1 induction which leads to elevated reactive oxygen species (ROS) generation, which in turn induces hepatic liver injury via ROS-mediated cell death. An inverse agonist of ERR, GSK5182, ameliorates alcoholic liver injury via inhibition of CYP2E1-mediated oxidative stress. Error bars show $\pm$ SEM * p <0.05, ** p <0.01, *** p <0.001 by two-tailed Student t test. Different lowercase letters in (D) show statistical significance (p <0.05) by one-way ANOVA among groups.