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Cytochrome P450 2E1 dependent hepatic ethanol metabolism induces fatty acid binding protein 4 and steatosis

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Abstract

Background.—Hepatic steatosis is an early pathology of alcohol-associated liver disease (ALD). Fatty acid binding protein-4 (FABP4, an FABP not normally produced in the liver) is secreted by hepatocytes in ALD and stimulates hepatoma proliferation and migration. This study sought to investigate mechanism[s] by which hepatic ethanol (EtOH) metabolism regulates FABP4 and steatosis.

Methods—Human hepatoma cells (HepG2/HuH7), and cells transfected to express cytochrome P450 2E1 (CYP2E1), were exposed to EtOH in the absence or presence of chlormethiazole (CYP2E1-inhibitor; CMZ) and/or EX-527 (sirtuin-1 [SIRT1] inhibitor). Culture medium was analyzed for EtOH metabolism and FABP4 protein abundance. Cells were analyzed for *FABP4* mRNA expression, SIRT1 protein abundance, and neutral lipid accumulation. In parallel, cells were analyzed for forkhead box O1 [FOXO1], β-catenin, peroxisome proliferator-activated receptor-α [PPARα], and lipin-1α protein abundance in the absence or presence of EtOH and pharmacological inhibitors of respective target proteins.

Results—CYP2E1-dependent EtOH metabolism inhibited the amount of SIRT1 protein detected, concomitant with increased *FABP4* mRNA expression, FABP4 protein secretion, and neutral lipid accumulation, effects abolished by CMZ. Analysis of pathways associated with lipid oxidation revealed increased FOXO1 nuclear localization and decreased β-catenin, PPARα and lipin-1α protein levels in CYP2E1-expressing cells in the presence of EtOH. Pharmacological inhibition of SIRT1 mimicked the effects of EtOH, while inhibition of FOXO1 abrogated the effect of EtOH on *FABP4* mRNA expression, FABP4 protein secretion, and neutral lipid accumulation in CYP2E1-expressing cells. Pharmacological inhibition of β-catenin, PPARα, or lipin-1α failed to alter the effects of EtOH on FABP4 or neutral lipid accumulation.

Conclusion—CYP2E1-dependent EtOH metabolism inhibits SIRT1-FOXO1 signaling leading to increased *FABP4* mRNA expression, FABP4 protein secretion, and neutral lipid accumulation.

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These data suggest FABP4 released from steatotic hepatocytes may play a role in promoting tumor cell expansion in the setting of ALD, and represents a potential target for therapeutic intervention.

Keywords

Ethanol; Cytochrome P450 2E1; Steatosis; Fatty acid binding protein-4 (FABP4); Sirtuin-1

INTRODUCTION

Hepatic alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) are the major enzymatic pathways for ethanol (EtOH) metabolism. Following moderate intake, ADH uses nicotinamide adenine dinucleotide (NAD^+) as a cofactor to rapidly oxidize EtOH to acetaldehyde and NADH (Cederbaum, 2012). In the setting of sustained, heavy EtOH consumption CYP2E1 is induced to oxidize EtOH to acetaldehyde, converting reduced NAD phosphate (NADPH) to its oxidized form ($NADP^+$) in the process (Seitz, 2020). In addition to the toxic effects of acetaldehyde, CYP2E1-dependent EtOH metabolism also elevates reactive oxygen species (ROS) production while depleting antioxidant levels to further damage cellular proteins, lipids, and nucleic acids (Cederbaum, 2010, Seitz, 2020).

The term alcohol-associated liver disease (ALD) covers a broad spectrum of liver pathologies, of which steatosis (increased fat accumulation) is the earliest and most common response (You and Arteel, 2019). If sustained heavy EtOH consumption continues, there is an increased susceptibility to progress to fibrotic liver disease (cirrhosis) (Ishak et al., 1991). Under normal circumstances hepatic fat transport and storage occurs *via* a number of integrated pathways, including a central role for fatty acid binding proteins (FABPs) (Ockner et al., 1982, Thumser et al., 2014). Functionally, FABPs bind free fatty acids (FFAs) with varying affinities, mechanisms, and ligand preference to regulate transport, metabolism, and storage (Thumser et al., 2014). To date, nine mammalian FABPs have been identified, and subtype expression is predominantly tissue specific, FABP1 being the form expressed in hepatocytes (Ockner et al., 1982).

Fatty acid binding protein 4 (FABP4) is usually expressed in adipocytes and macrophages (Hunt et al., 1986, Makowski et al., 2001). However, increasing evidence suggests FABP4 can be synthesized and released by adipocytes in specific cancers to function as a paracrine/endocrine signaling factor. For example, hepatocellular carcinoma (HCC) patients with metabolic syndrome are characterized by elevated endothelial FABP4 protein release that promotes carcinogenesis (Laouirem et al., 2019). Similarly, obesity and alcohol-induced hepatosteatosis induces FABP4 protein synthesis and release from hepatocytes (Attal et al., 2021, Thompson et al., 2018), while exogenous recombinant human FABP4 protein stimulates HCC proliferation and migration in hepatoma cells *in vitro* (Attal et al., 2021). Conversely, low levels of FABP4 are reported in HCCs in patients with underlying viral hepatitis B (HBV) infection, while overexpressing FABP4 in HBV^+ HCC cells inhibits proliferation *in vitro* and tumor formation in a xenograph model *in vivo* (Zhong et al., 2018).

A central role for sirtuin-1 (SIRT1; a nuclear NAD^+-dependent protein deacetylase (Smith et al., 2000)) is reported in the regulation of transcriptional networks involved in lipid metabolism in hepatocytes (Ding et al., 2017, Purushotham et al., 2009), and

Sirt-1 is implicated in regulating FABP4 abundance in adipocytes (Josephrajan et al., 2019). Functionally, SIRT1 activates (β-catenin, peroxisome proliferator-activated receptor-α [PPAR-α]/PPAR-γ coactivator1-α [PGC1-α], lipin-1α) or represses (forkhead Box O1 [FOXO1], acetyl-CoA carboxylase 1/2 [ACC1/2], sterol regulatory-element binding protein [SREBP]) transcription factors to maintain lipid homeostasis, such that decreased SIRT1 activity reduces the deacetylation of proteins involved in decreasing free fatty acid oxidation and/or increases lipogenesis, resulting in net lipid accumulation (Ding et al., 2017, Guclu et al., 2016).

Following ethanol metabolism to acetaldehyde, acetaldehyde is oxidized in the mitochondria to acetate *via* aldehyde dehydrogenase and the reduction of NAD^+ to NADH (Cederbaum, 2012). In parallel, sustained, heavy ethanol intake impairs hepatic mitochondrial oxidative phosphorylation by suppressing synthesis of subunits of the respiratory complexes, including NADH dehydrogenase (Complex I) (Manzo-Avalos and Saavedra-Molina, 2010, Cunningham et al., 1990). These consequences of EtOH metabolism on intracellular NAD^+/NADH levels can be further exacerbated by mitochondrial damage arising due to increased intracellular ROS production (Manzo-Avalos and Saavedra-Molina, 2010, Cahill and Cunningham, 2000). Given the impact of EtOH metabolism on hepatic NAD^+/NADH, and the role of NAD^+ in SIRT1 activity, we sought to investigate the potential role of SIRT1 signaling in changes in hepatic FABP4 and steatosis during hepatic EtOH metabolism.

MATERIALS AND METHODS

Cell culture.

Human HepG2 HCC cells were purchased from ATCC (Manassas, VA) and human HuH7 HCC cells from the JCRB Cell Bank (Sekisui XenoTech; Kansas City, KS). HepG2 and HuH7 cells stably transfected to express CYP2E1 (E47 (Chen and Cederbaum, 1998) and $HuH7^{CYP+}$ (Osna et al., 2008)) were generous gifts from Dr A. Cederbaum (Ichan School of Medicine, NY) and Dr N. Osna (University of Nebraska Medical Center, Omaha, NE) respectively.

Ethanol metabolizing assay:

HepG2/E47 and HuH7/$HuH7^{CYP+}$ were maintained in low serum culture medium (LSM; 0.1% [*v/v*] fetal bovine serum (FBS) Dulbecco's Modified Eagle Medium) for 24-hours prior to exposure to EtOH (50mM, 48-hours). Culture medium was collected and EtOH measured using an Ethanol Assay Kit (BioAssay Systems, Hayward, CA).

mRNA analysis.

HepG2/E47 and HuH7/$HuH7^{CYP+}$ cells were maintained in LSM for 24-hours prior to exposure to EtOH (0-100mM, 48-hours). Total RNA extraction was performed using a Quick-RNA™ Miniprep kit (Zymo Research, Tustin, CA), and reverse transcribed to cDNA using an IMPROM II™ transcription system (Promega, Madison, WI). Quantitative RT-PCR (qRT-PCR) was performed using a TaqMan® Universal Master Mix II kit and TaqMan probes for FABP4 and SIRT1 (Thermo Fisher Scientific, Grand Island, NY). *FABP4* and *SIRT1* mRNA expression were calculated and normalized to 18s RNA.

FABP4 protein detection.

HepG2/E47 and HuH7/HuH7^{CYP+} were maintained in LSM for 24-hours prior to exposure to EtOH (0-100mM, 48-hours). Culture medium was collected and FABP4 protein abundance was measured using an enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) (Attal et al., 2021). In parallel, identical studies were performed in which cells were exposed to EtOH (50mM) in the absence or presence of the CYP2E1 inhibitor chlormethiazole (CMZ, 100μM, Sigma-Aldrich, St. Louis; MO) (Hu et al., 1994).

Acetaldehyde generating system.

To study the effect of acetaldehyde on *FABP4* mRNA expression and FABP4 protein secretion, HepG2 and HuH7 cells were exposed to an acetaldehyde generating system (48-hours) comprising of 0.2U/mL yeast alcohol dehydrogenase (ADH), 2mM β-nicotinamide adenine dinucleotide (β-NAD), and 50mM EtOH (Sigma-Aldrich) (Ganesan et al., 2015). At completion, cells and culture medium were collected and analyzed for *FABP4* mRNA expression (qRT-PCR) and FABP4 protein secretion into the culture medium (ELISA).

Role of ethanol metabolism in regulating SIRT1 and FABP4.

HepG2/E47 and HuH7/HuH7^{CYP+} cells were maintained in LSM for 24-hours prior to exposure to CMZ (100μM, 2Hrs) followed by exposure to EtOH (0-100mM, 48-hours). Culture medium was removed, cell lysates prepared in radioimmuno precipitation assay (RIPA) buffer containing protease/phosphatase inhibitors and the amount of SIRT1 protein was detected by Western blot. In parallel, cells were treated with EtOH (0-100mM) in the absence or presence of a SIRT1 inhibitor (EX-527, 50μM, Sigma-Aldrich) (Li et al., 2016) and cells/culture medium collected 48-hours later and analyzed for *FABP4* mRNA expression (qRT-PCR) and FABP4 protein secretion (ELISA).

SIRT1 Over-expression.

HepG2/E47 and HuH7/HuH7^{CYP+} cells were grown to 80% confluency prior to transfection using a pCMV6-AC-GFP plasmid expressing the *SIRT1* gene (NM_012238; Origene Technologies Rockville, MD) using Lipofectamine 3000 Reagent (Thermo Fisher Scientific). Transfection efficiency was established microscopically by visualizing green fluorescent protein (GFP) co-expressed with the *SIRT1* gene. Cells were exposed to EtOH (50mM) and cells/culture medium collected 48-hours later and analyzed for *FABP4* mRNA expression (qRT-PCR), FABP4 protein secretion (ELISA), and SIRT1/GFP abundance (Western blot).

Mechanisms CYP2E1-catalyzed ethanol metabolism and SIRT1-regulated free fatty acid oxidation.

HepG2/E47 and HuH7/HuH7^{CYP+} cells were maintained in LSM for 24-hours prior to exposure to EtOH (50mM, 48-hours). For the analysis of FOXO1/phospho-FOXO1, nuclear and cytoplasmic fractions were extracted using NE-PER™ nuclear and cytoplasmic extraction reagents according to the manufacturer's directions (Pierce Biotechnology, Rockford; IL). Total protein concentrations were determined, protein concentrations adjusted to equivalence, and samples analyzed by Western blot using antibodies against

FOXO1/pFOXO1 (Cell Signaling Technologies). For samples analyzed using anti-FOXO1, 10μg of total protein was loaded in each lane. For samples analyzed using anti-pFOXO1, 40μg of total protein was loaded in each lane. Because different protein amounts were required for detection using each antibody, samples were run independently rather than stripping the membranes/re-probing them. For all other analyses, Western blots were performed using antibodies against β-catenin and PPARα (Cell Signaling Technologies) or lipin-1α (Abcam, Waltham, MA). Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD) and protein correction performed to Ponceau S staining.

Effect of CYP2E1-catalyzed ethanol metabolism on SIRT1 regulation of downstream free fatty acid oxidation protein targets, FABP4 production, and lipid accumulation.

HepG2/E47 and HuH7/HuH7^{CYP+} cells were maintained in LSM for 24-hours prior to exposure to EtOH (50mM, 48-hours) in the absence or presence of inhibitors of FOXO1 (AS1842856; 1μM) (Zou et al., 2014), β-catenin (PKF118-310; 1μM) (Wei et al., 2010), PPARα (GW6471; 1μM) (Tachibana et al., 2018), or lipin-1α (propranolol hydrochloride [PHC]; 10μM) (Farah et al., 2014) (Sigma Aldrich). Cells and culture medium were collected 48-hours later and analyzed for *FABP4* mRNA expression (qRT-PCR) and FABP4 protein secretion into the culture medium (ELISA). In parallel, HepG2/E47 and HuH7/HuH7^{CYP+} cells were seeded in one-well Nunc Lab-Tek chamber slides (Thermo-Fisher Scientific) and maintained in LSM for 24-hours prior to EtOH exposure (50mM, 48-hours) in the absence/presence of AS1842856 (1μM), PKF118-310 (1μM), GW6471 (1μM), or PHC (10μM) for 48-hours.

Analysis of neutral lipid accumulation was performed using an Oil Red O staining kit (Bio vision, Milpitas, CA). Briefly, culture medium was removed, cells washed with phosphate buffered saline, fixed with 10% (*v/v*) neutralized formalin buffer (30-minutes, room temperature [RT]), and processed using 60% (*v/v*) isopropanol (5-minutes, RT). Cells were stained with an Oil Red O working solution (15-minutes, RT) followed by counterstain with hematoxylin (1-minute, RT). Lipid droplets (red) and nuclei (blue) were viewed with an inverted microscope and representative images captured. For quantification, cells were exposed to 100% isopropanol (5-minutes, RT) and absorbance measured at 492nm (Ali et al., 2018).

Quantification and statistical Analysis.

All experiments were performed a minimum of three times and data are expressed as mean ± SEM. One- and two-way ANOVA were performed as appropriate using open-source R statistical software (version 3.5.3). A p-value <0.05 was considered statistically significant.

RESULTS

CYP2E1-dependent ethanol metabolism increases FABP4.

Western blot confirmed the absence of CYP2E1 and ADH protein in HepG2 and HuH7 cells and the inability of these cells to metabolize EtOH (Supplemental Figure 1A-C). Conversely, CYP2E1 protein was readily detectable in the E47 and HuH7^{CYP+}, and both cell lines metabolized EtOH (Supplemental Figure 1A-C). Analysis of *FABP4* mRNA expression

and FABP4 secretion into culture medium in the presence of EtOH revealed no change in HepG2 or HuH7 cells (Supplemental Figure 1D & E). Conversely, exposure to EtOH led to concentration dependent increases in FABP4 mRNA expression and FABP4 protein levels in culture medium in E47 and HuH7^{CYP+} cells (Supplemental Figure 1D & E).

Using an acetaldehyde generating system, EtOH failed to alter *FABP4* mRNA expression or FABP4 protein levels in culture medium in HepG2 or HuH7 cells (Figure 1A and B, N=3 independent experiments). Conversely, exposure of E47 and HuH7^{CYP+} cells to EtOH led to increased *FABP4* mRNA expression and FABP4 protein abundance in culture medium, effects that were inhibited by CMZ (Figure 1C and D, N=3 independent experiments, $p<0.05$ 0mM EtOH *versus* 50mM EtOH, $p<0.05$ 50mM EtOH *versus* 100μM CMZ + 50mM EtOH).

CYP2E1-dependent ethanol metabolism leads to accumulation of intracellular neutral lipids.

Exposure of HepG2 and HuH7 cells to EtOH, or EtOH in the presence of CMZ, failed to alter intracellular neutral lipid accumulation (data not shown). In contrast, EtOH led to increased intracellular neutral lipid accumulation in both E47 and HuH7^{CYP+} cells, effects that were abrogated by CMZ (Figure 1E & F, N=3 independent experiments, $p<0.05$ 0mM EtOH *versus* 50mM EtOH, $p<0.05$ 50mM EtOH *versus* 100μM CMZ + 50mM EtOH).

CYP2E1-dependent ethanol metabolism inhibits SIRT1, alters FABP4 expression, and increases intracellular neutral lipid accumulation.

Exposure of HepG2 and HuH7 cells to EtOH failed to alter SIRT1 protein expression (data not shown). Conversely, exposure of E47 and HuH7^{CYP+} cells to EtOH led to a concentration dependent decrease in SIRT1 protein abundance (Figures 2A-D, N=3 independent experiments, $p<0.05$ *versus* 0mM EtOH), effects that were abrogated by CMZ (Figures 2A-D, N=*3* independent experiments, $p<0.05$ *versus* 0mM EtOH). Exposure of E47 and HuH7^{CYP+} cells to EtOH led to increased *FABP4* mRNA expression and FABP4 protein abundance in the culture medium, effects that were mimicked by inhibiting SIRT1 with EX-527 (Figure 3A & B, N=3 independent experiments, $p<0.05$ 0mM EtOH *versus* 50mM EtOH, $p<0.05$ 0mM EtOH *versus* 50μM EX-527 + 50mM EtOH).

To confirm a role for EtOH metabolism and SIRT1, cells were transfected with a plasmid expressing the *SIRT1* and *GFP* genes (Supplemental Figure 2). Following over-expression of *SIRT1* in E47 and HuH7^{CYP+} cells EtOH failed to induce FABP4 mRNA expression or elevate FABP4 protein abundance in the culture medium (Figure 3C & D, N=3 independent experiments, $p<0.05$ 0mM EtOH *versus* 50mM EtOH, $p<0.05$ 50mM EtOH *versus* SIRT1^{+} cells + 50mM EtOH). In cells that do not express CYP2E1 (HepG2 and HuH7), EtOH failed to stimulate *FABP* mRNA expression or FABP4 protein abundance in culture medium, and this did not change in SIRT1^{+} HepG2 and HuH7 cells exposed to EtOH (50mM) (data not shown).

Downstream effectors of SIRT1; regulation of FABP4 expression and cellular steatosis.

FOXO1.—Exposure of E47 and HuH7^{CYP+} cells to EtOH reduced the amount of pFOXO1 protein detected in the cytoplasmic fraction concomitant with an increased

amount of FOXO1 protein detected in the nuclear fraction (Figure 4A-D, N=3 independent experiments, p<0.05 0mM EtOH *versus* 50mM EtOH). In contrast, HepG2 and HuH7 exposed to EtOH did not result in changes in FOXO1 protein phosphorylation state or localization (Figure 4A-D). Exposure of cells to AS184 3856 (AS) prior to EtOH abrogated the effect of EtOH on *FABP4* mRNA expression, amount of FABP4 protein detected in the culture medium, and neutral lipid accumulation (Figure 5A-D, N=3 independent experiments, p<0.05 0mM EtOH *versus* 50mM EtOH, p<0.05 50mM EtOH *versus* 1μM AS + 50mM EtOH). Using HepG2 and HuH7 cells, EtOH (50mM) failed to alter *FABP4* mRNA expression, amount of FABP4 protein detected in the culture medium, or neutral lipid accumulation, and this remained so in the presence of AS (data not shown).

β-catenin.—Exposure of E47 and HuH7^{CYP+} to EtOH inhibited the amount of β-catenin protein detected, effects that were not evidenced in HepG2 or HuH7 cells (Supplemental Figure 3A & B, N=3 independent experiments, p<0.05 0mM EtOH *versus* 50mM EtOH). Exposure of cells to PKF118-310 (PKF) prior to EtOH did not alter the effect of EtOH on *FABP4* mRNA expression, amount of FABP4 protein detected in the culture medium, or neutral lipid accumulation (Supplemental Figure 3C-F, N=3 independent experiments, p<0.05 *versus* 0mM EtOH). Using HepG2 and HuH7 cells, EtOH (50mM) failed to alter *FABP4* mRNA expression, amount of FABP4 protein detected in the culture medium, or neutral lipid accumulation, and this remained so in the presence of PKF (data not shown).

PPARα.—Exposure of E47 and HuH7^{CYP+} cells to EtOH inhibited the amount of PPARα protein detected, effects that were not evidenced in HepG2 or HuH7 cells (Supplemental Figure 4A & B, N=3 independent experiments, p<0.05 0mM EtOH *versus* 50mM EtOH). Exposure of cells to GW6471 (GW) prior to EtOH not alter the effect of EtOH on *FABP4* mRNA expression, FABP4 protein detected in the culture medium, or neutral lipid accumulation (Supplemental Figure 4C-F, N=3 independent experiments, p<0.05 *versus* 0mM EtOH). Using HepG2 and HuH7 cells, EtOH (50mM) failed to alter *FABP4* mRNA expression, FABP4 protein detected in the culture medium, or neutral lipid accumulation, and this remained so in the presence of GW (data not shown).

Lipin-1.—Exposure of E47 and HuH7^{CYP+} to EtOH inhibited the amount of lipin-1 protein detected, effects that were not evidenced in HepG2 or HuH7 cells (Supplemental Figure 5A & B). Exposure of cells to propranolol hydrochloride (PHC) prior to EtOH did not alter the effect of EtOH on *FABP4* mRNA expression, FABP4 protein detected in the culture medium, or neutral lipid accumulation (Supplemental Figure 5C-F, N=3 independent experiments, p<0.05 *versus* 0mM EtOH). Using HepG2 and HuH7 cells, EtOH (50mM) failed to alter *FABP4* mRNA expression, FABP4 protein detected in the culture medium, or neutral lipid accumulation, and this remained so in the presence of PHC (data not shown).

DISCUSSION

The persistent metabolic stress and cell damage resulting from steatosis in ALD induces a sustained immune response and augments the wound-repair response toward a fibrosis/cirrhosis. Data presented herein demonstrate CYP2E1-dependent EtOH metabolism inhibits the amount of SIRT1 detected, induces *FABP4* mRNA expression and FABP4 protein

secreted into the culture medium, and leads to increased neutral lipid accumulation, effects that are abrogated by inhibiting CYP2E1 (CMZ) and mimicked by transfecting cells to over-express SIRT1. Analysis of pathways downstream of SIRT1 suggest SIRT1-FOXO1 signaling underlies the effects of CYP2E1-EtOH metabolism on FABP4 and cellular steatosis (Figure 6).

Significant progress has been made understanding the role of CYP2E1 in ALD progression. Using CYP2E1 knockout mouse models ($CYP2E1^{-/-}$), and humanized CYP2E1 knock-in mice, the effects of high fat-EtOH feeding on steatosis in $CYP2E1^{-/-}$ mice are blunted and can be reversed by re-introducing CYP2E1 (Cederbaum, 2010). Similarly, EtOH-induced steatosis is reversed by CMZ in chronic-EtOH fed mice (Lu et al., 2008), and blunted in an intragastric EtOH (IG-EtOH) feeding model (Gouillon et al., 2000). However, in other intragastric EtOH feeding studies, either no differences in steatosis were observed between $CYP2E1^{-/-}$ and wild type mice (4-week feeding) (Kono et al., 1999), or that steatosis was observed in only $CYP2E1^{-/-}$ mice (3-week feeding) (Wan et al., 2001). These data suggest both the feeding model and the compensatory mechanism[s] that arise in genetically modified models are important factors in interpreting the role of CYP2E1 in EtOH-induced steatosis.

Sirtuin-1 is a focal point for understanding the changes in intracellular signaling following EtOH-dependent CYP2E1 induction. Previous studies report CYP2E1-catalyzed EtOH metabolism increases ROS production and suppresses SIRT1 expression *in vitro* (Nagappan et al., 2018). These data are supported *in vivo* using hepatocyte specific SIRT1 knockout mice whereby SIRT1 deletion promotes EtOH-dependent steatosis, inflammation, and fibrosis (Yin et al., 2014). These findings are mirrored in our studies where inhibition of SIRT1 mimicked the effects of EtOH in CYP2E1 expressing cells to promote steatosis and increase the amount of FABP4 released into culture medium, while over-expression of SIRT1 abolished the effect of EtOH in CYP2E1-expressing cells.

The role of SIRT1 in steatosis is dependent on the interaction of SIRT1 with several potential downstream transcription factors that regulate free fatty acid synthesis (lipogenesis) and metabolism (β-oxidation) (Ding et al., 2017, Purushotham et al., 2009, Ren et al., 2020). A net increase in intracellular lipids induces genetic responses to regulate fatty acid transporter expression (including FABPs) to sequester free fatty acids for oxidation and/or storage (Smathers et al., 2011). This is evidenced in adipocytes, whereby exposure to nicotinamide (SIRT1 inhibitor), or employing a SIRT1-siRNA approach, induces FABP3 expression (Shan et al., 2009). Similarly, activation of SIRT1 using oxymatrine (hepatoprotective alkaloid) in a rat model of non-alcoholic steatosis decreases the expression of genes involved in lipogenesis concomitant with decreased FABP1 expression, reduced body/liver weight, and decreased hepatic triglyceride content (Xu et al., 2020). Conversely, others report FABP4 is unconventionally secreted from adipocytes in a SIRT1-dependent manner in response to lipolytic stimulation (Josephrajan et al., 2019). Explanations for discrepancies in how SIRT1 may regulate FABP production and steatosis likely relate to the ability of SIRT1 to regulate multiple downstream pathways (Ding et al., 2017).

In our studies, we identify a link between CYP2E1-EtOH metabolism, inhibition of SIRT1-FOXO1 protein abundance, changes in FABP4 protein secretion, and steatosis. Intracellularly, FOXO1 activity is regulated by subcellular localization following phosphorylation/acetylation (inactive; cytoplasm) and dephosphorylation/deacetylation (active; nucleus), such that increased SIRT1 activity inactivates FOXO1 to target it to the cytoplasm for degradation (Wang et al., 2011). This is evidenced *in vivo,* where hepatic tissue from liver specific SIRT1$^{-/-}$ mice are characterized by decreased FOXO1 phosphorylation and increase FOXO1 target gene expression, while transgenic mice that over-express constitutively active (nuclear) FOXO1 exhibit hepatic steatosis (Lieber et al., 2008). Inhibition of FOXO1 with siRNA reduces FABP4 while constitutively active nuclear FOXO1 increases FABP4 (Harjes et al., 2014). Similarly, phosphorylation of FOXO1 by apelin (fatty acid transport inhibitor) leads to decreased amounts of FABP4 (Hwangbo et al., 2017). However, our evidence indicating a role for SIRT1-FOXO1 signaling in regulating *FABP4* mRNA expression and protein secretion should not discount the potential role of other SIRT1 targets.

Impaired β-catenin signaling is important in several forms of liver injury. Liver specific β-catenin$^{-/-}$ mice maintained on alcohol diets display redox imbalance, impaired hepatic fatty acid oxidation, and severe steatosis concomitant with decreased amounts of SIRT1 (Lehwald et al., 2012). β-Catenin is also implicated in the regulation of FABP expression in adipocytes and macrophages to repress adipogenesis by inducing FABP4 protein production and activating proteasomal PPARγ degradation (Garin-Shkolnik et al., 2014), while FABP1 and FABP5 is reduced in liver specific β-catenin$^{-/-}$ mice (Lemberger et al., 2018). An integral role for SIRT1-lipin-1 signaling is also described in EtOH-dependent steatosis, with EtOH exposure inhibiting sumoylation of Lipin-1α (required for nuclear translocation) to prevent lipin-1α interaction with PPARα and PPARγ, resulting in increased fatty acid oxidation (Fischer et al., 2003) and favoring cytoplasmic lipin-1β localization and excess lipid accumulation in cultured hepatic cells and mouse liver (Lemberger et al., 2018). The role of lipin-1 in adipogenesis is inherently linked to the regulation of PPARα and PPARγ, with lipin-1 depletion in 3T3-L1 adipocytes resulting in lower amounts of PPARγ and FABP4 protein, while FABP1 directly interacts with PPARα in cultured hepatocytes to promote hepatic fatty acid β-oxidation (Hostetler et al., 2009).

When considering the complexities associated with SIRT1 signaling in fatty acid homeostasis a number of factors must be considered that include the cell type (*e.g.* adipocytes *versus* hepatocytes), whether studies are performed *in vitro* or *in vivo*, the nature of the model employed (genetic/cell specific knockout, over expression system, or pharmacological), and the underlying pathology (*e.g.* ethanol *versus* obesity). This may be particularly relevant in our model system since the HepG2 and HuH7 cell lines were originally derived from human hepatomas and are thus fundamentally different to hepatocytes. Similarly, the cells we used were only transfected to express CYP2E1 and do not express ADH, whereas hepatocytes in human ALD and rodent models of chronic EtOH ingestion have both ADH and CYP2E1 present. This may have particular significance given the difference in catalytic efficiency for EtOH between ADH and CYP2E1 (Cederbaum, 2012), and the role of NAD^+ as a cofactor for both ADH and SIRT1 (Cederbaum, 2012, Smith et al., 2000) .

Previous studies report increased hepatic *FABP4* mRNA expression in hepatocytes derived from mouse models of ALD and in human liver tissue from ALD patients. Furthermore, exposure of HCC cells to exogenous recombinant human FABP stimulates HCC proliferation and migration (Attal et al., 2021). While additional work is required to determine the relative contribution of ADH *versus* CYP2E1 in regulating hepatic FABP4, the intriguing possibility is raised that chronic EtOH exposure stimulates hepatocyte FABP4 synthesis and release as part of the steatotic response. In doing so, the released FABP4 may in turn function to promote transformed (tumor) cell expansion within the hepatic microenvironment.

When interpreting our data, it is important to highlight limitations to these studies. Firstly, our focus was to study potential mechanisms whereby EtOH metabolism alters hepatic *FABP4* mRNA expression and/or protein secretion. Doing so used hepatoma cell lines stably transfected to express CYP2E1 (Chen and Cederbaum, 1998, Osna et al., 2017), and these cells are fundamentally different from hepatocytes *in vivo*. Secondly, while our data support the involvement of CYP2E1-dependent EtOH metabolism in promoting increased *FABP4* mRNA expression and the amount of FABP4 protein secreted, the role of ADH in these events should not be discounted. Our studies employed an artificial, and thus non-physiological, acetaldehyde generating system. This system has previously been described and used by Ganesan and colleagues with HuH7^{CYP+} cells, and is reported to generate acetaldehyde levels that fluctuate between 250μM (1-4 hours of exposure) and 50μM (18-48 hours of exposure) (Ganesan et al., 2015), levels that correspond to the amount of acetaldehyde produced by ADH expressing liver cells (Donohue et al., 2006). However, it should be noted that the kinetics of acetaldehyde production in this system may differ to that of ADH expressing cells, and it would be of interest to further refine our studies using HepG2 cells that have been stably transfected to express ADH (VA-13 cell line (Clemens et al., 2002)) or ADH and CYP2E1 (VL-17A cell line (Donohue et al., 2006)). Finally, our studies employed pharmacological inhibitors to study SIRT1 signaling pathways. As with all studies using such inhibitors there is an inherent risk of non-specific intracellular effects. However, this approach has allowed us to refine pathways of interest from which future studies using specific molecular biology approaches (shRNA/siRNA or CRISPR/Cas9) can be developed.

CONCLUSION

Cytochrome P450 2E1-dependent EtOH metabolism inhibits a SIRT1-FOXO1 pathway leading to increased *FABP4* mRNA expression, FABP4 protein secretion into the culture medium, and neutral lipid accumulation *in vitro*. In conjunction with previous studies demonstrating FABP4 stimulation of HCC proliferation and migration, these data may prove insightful in further our understanding of potential mechanisms of HCC progression in the setting of ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Support

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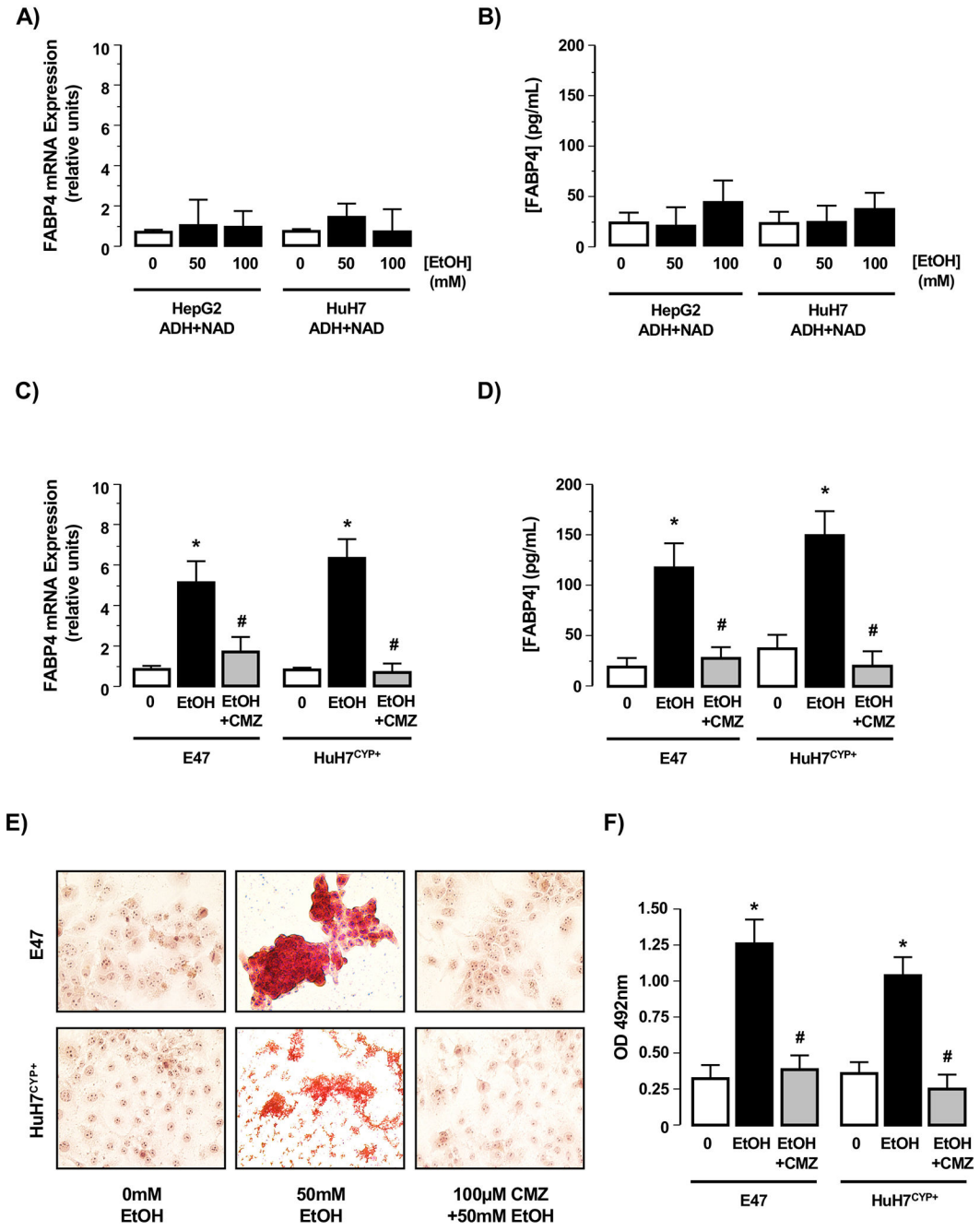

Figure 1.

Cytochrome P4502E1 (CYP2E1) dependent ethanol metabolism alters* FABP4 *mRNA expression and FABP4 protein secretion. HepG2 and HuH7 cells were exposed to 0, 50, or 100mM ethanol (EtOH) in the presence of an acetaldehyde generating system and ***A)*** *Fatty acid binding protein 4* (*FABP4*) mRNA expression and ***B)*** FABP4 protein secretion into culture medium measured. N=3 independent experiments. Effect of 0 or 50mM EtOH on HepG2 and HuH7 cells transfected to express CYP2E1 (E47 and HuH7^{CYP+}) in the absence or presence of chlormethiazole (CMZ; 100μM) on ***C)*** *FABP4* mRNA expression and ***D)*** FABP4 protein secretion into culture medium. N=3 independent experiments, *p<0.05

50mM EtOH *versus* 0mM EtOH, $^{\#}p<0.05$ CMZ + 50mM EtOH *versus* 50mM EtOH. ***E)*** Representative images of E47 and HuH7^{CYP+} cells exposed to 0 or 50mM EtOH in the absence or presence of CMZ (100μM) following Oil red-O staining to detect neutral lipids. ***F)*** Quantification of Oil red-O staining by optical density (OD) absorption (492nm). N=3 independent experiments, *p<0.05 50mM EtOH *versus* 0mM EtOH, $^{\#}p<0.05$ CMZ + 50mM EtOH *versus* 50mM EtOH.

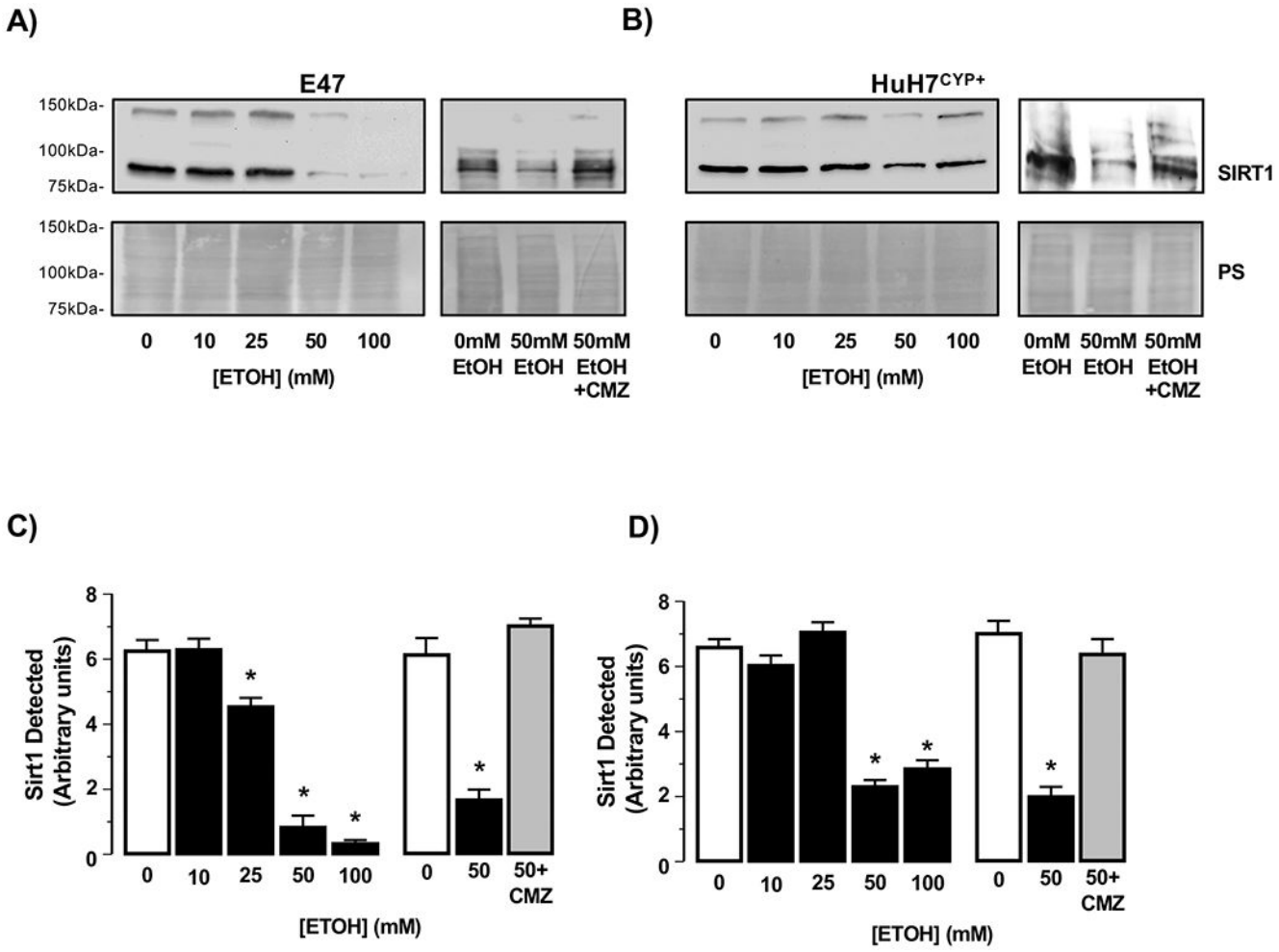

Figure 2. Cytochrome P4502E1-dependent ethanol metabolism inhibits sirtuin-1 expression.

A) Representative Western blot analysis of amount of sirtuin-1 (SIRT1) detected in HepG2 cells transfected to express CYP2E1 (E47) following exposure to ethanol (EtOH; 0-100mM) or exposure to EtOH (50mM) in the absence or presence of chlormethiazole (CMZ; 100μM). Equal protein loading was assessed by Ponceau-S (PS) membrane stain. ***B)*** Representative Western blot analysis of amount of SIRT1 detected in HuH7 cells transfected to express CYP2E1 (HuH7^{CYP+}) following exposure to EtOH (0-100mM) or exposure to EtOH (50mM) in the absence or presence of CMZ (100μM). Equal protein loading was assessed by PS membrane stain. ***C)*** Cumulative densitometric analysis of relative SIRT1 protein detected in E47 cells following exposure to EtOH (0-100mM), or exposure to EtOH (50mM) in the absence or presence of CMZ (100μM). N=3 independent experiments, *p<0.05 *versus* 0mM EtOH). ***D)*** Cumulative densitometric analysis of relative SIRT1 protein detected in HuH7^{CYP+} cells following exposure to EtOH (0-100mM), or exposure to EtOH (50mM) in the absence or presence of CMZ (100μM). N=3 independent experiments, *p<0.05 *versus* 0mM EtOH).

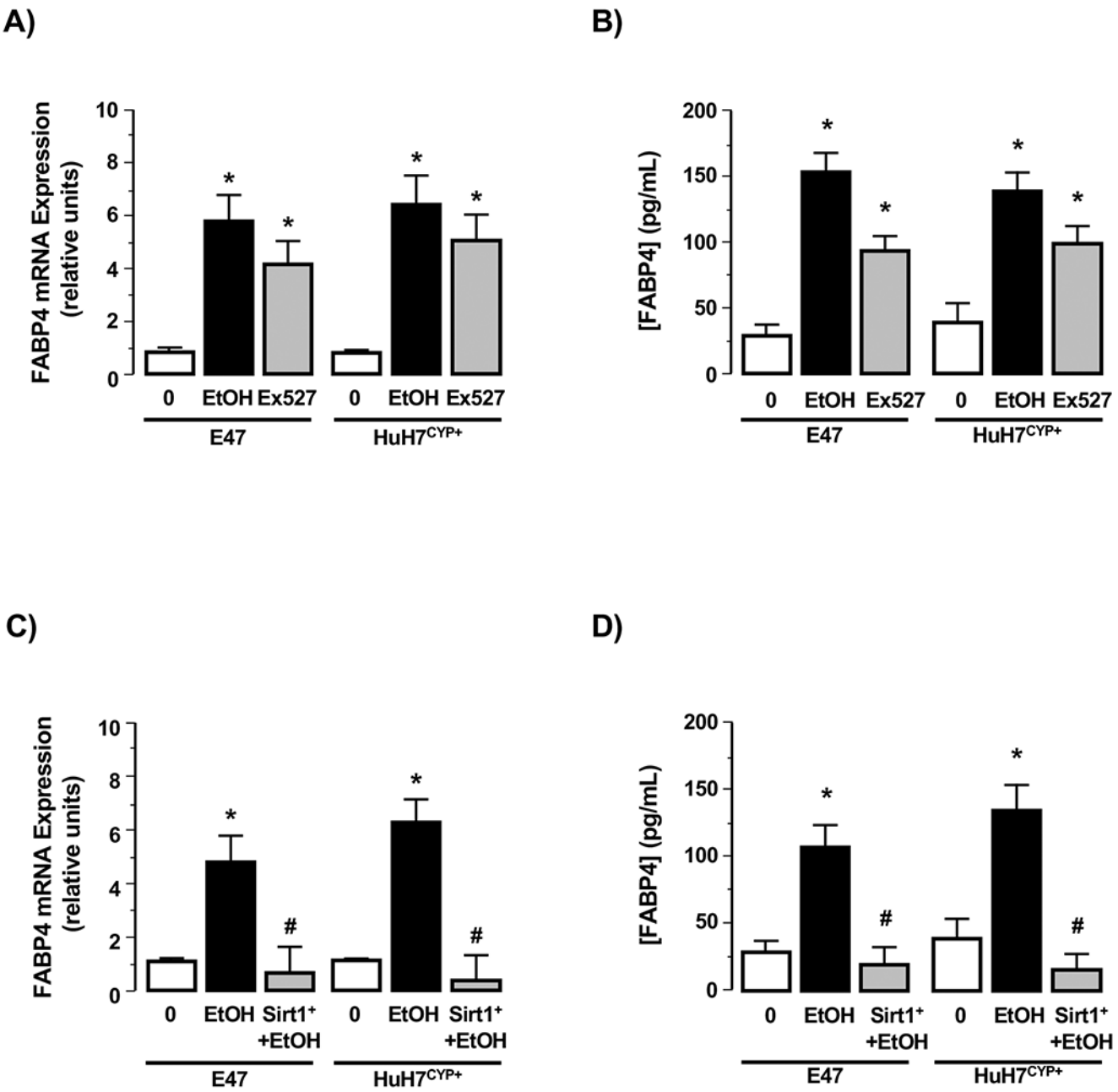

Figure 3. ***Altering sirtuin 1 activity/expression alters* FABP4 *mRNA expression and FABP protein secretion.***

A) Effect of EtOH (50mM) or Ex547 (SIRT1 inhibitor, 50μM) on *FABP4* mRNA expression in HepG2 and HuH7 cells transfected to express CYP2E1 (E47/HuH7^{CYP+}). N=3 independent experiments, *p<0.05 *versus* 0mM EtOH). ***B)*** Effect of EtOH (50mM) or Ex547 (50μM) on amount of FABP4 protein detected in culture medium in E47 and HuH7^{CYP+} cells. N=3 independent experiments, *p<0.05 *versus* 0mM EtOH). ***C)*** Effect of EtOH (50mM) on *FABP4* mRNA expression in E47 and HuH7^{CYP+} cells and E47 and HuH7^{CYP+} cells transfected to overexpress SIRT1 (SIRT1^{+}). N=3 independent experiments, *p<0.05 50mM EtOH *versus* 0mM EtOH, $^{\#}$p<0.05 SIRT1^{+} + 50mM EtOH *versus* 50mM

EtOH. ***D)*** Effect of EtOH (50mM) on FABP4 protein secretion into culture medium from E47 and HuH7^{CYP+} cells and SIRT1^{+} E47 and HuH7^{CYP+} cells. N=3 independent experiments, *p<0.05 50mM EtOH *versus* 0mM EtOH, $^{\#}$p<0.05 SIRT1^{+} + 50mM EtOH *versus* 50mM EtOH.

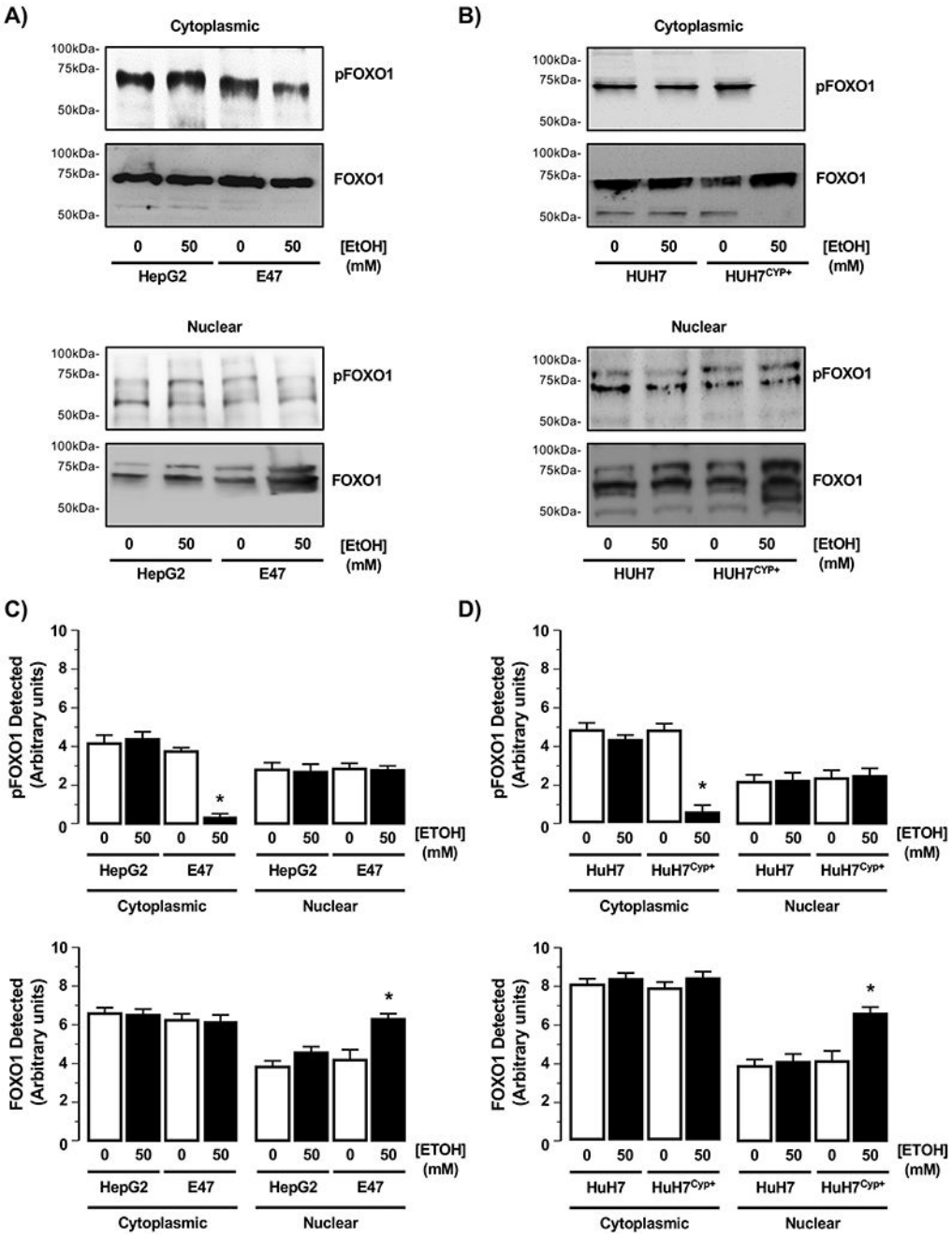

Figure 4. Cytochrome P4502E1-dependent ethanol metabolism induces dephosphorylation of FOXO1/nuclear localization.

A) Representative Western blot analysis of phosphorylated and dephosphorylated FOXO1 (pFOXO1/FOXO1) protein abundance in cytoplasmic and nuclear cell fractions in HepG2 cells and HepG2 cells transfected to express CYP2E1 (E47) following exposure to ethanol (EtOH; 50mM). ***B)*** Representative Western blot analysis of pFOXO1 and FOXO1 protein abundance in cytoplasmic and nuclear cell fractions in HuH7 cells and HuH7 cells transfected to express CYP2E1 ($HuH7^{CYP+}$) following exposure to EtOH (50mM). ***C)*** Cumulative densitometric analysis of relative pFOXO1 and FOXO1 protein detected in

cytoplasmic and nuclear cell fractions from HepG2 and E47 cells following exposure to EtOH (50mM). N=3 independent experiments, *p<0.05 *versus* 0mM EtOH. ***D)*** Cumulative densitometric analysis of relative pFOXO1 and FOXO1 protein detected in cytoplasmic and nuclear cell fractions from HuH7 and HuH7^{CYP+}cells following exposure to EtOH (50mM). N=3 independent experiments, *p<0.05 *versus* 0mM EtOH.

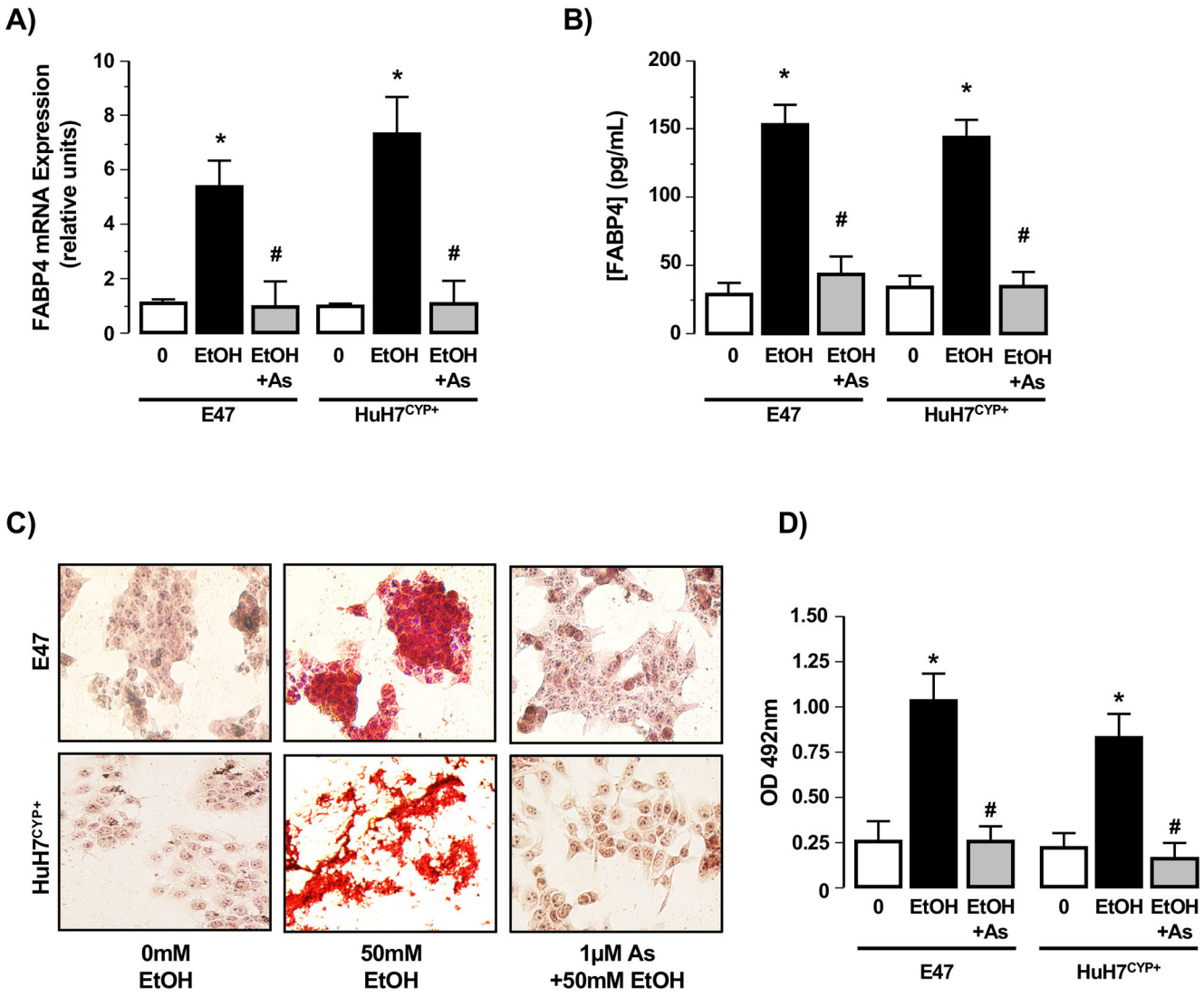

Figure 5. Inhibition of FOXO1 abrogates the effect of ethanol on FABP4 mRNA expression, FABP4 secretion and neutral lipid accumulation in CYP2E1 expressing cells.

A) Effect of ethanol (EtOH, 50mM) on *FABP4* mRNA expression in HepG2 and HuH7 cells transfected to express CYP2E1 (E47/HuH7^{CYP+}) in the absence or presence of the FOXO1 inhibitor AS184 3856 (AS; 1µM). N=3 independent experiments, *p<0.05 50mM EtOH *versus* 0mM EtOH, $^{\#}$p<0.05 AS + 50mM EtOH *versus* 50mM EtOH. ***B)*** Effect of EtOH (50mM) on FABP4 protein detected in culture medium from E47 and HuH7^{CYP+} cells in the absence or presence of AS (1µM). N=3 independent experiments, *p<0.05 50mM EtOH *versus* 0mM EtOH, $^{\#}$p<0.05 AS + 50mM EtOH *versus* 50mM EtOH. ***C)*** Representative images of E47 and HuH7^{CYP+} cells exposed to 0 or 50mM EtOH in the absence or presence of AS (1µM) following Oil red-O staining to detect neutral lipids. ***D)*** Relative quantification of Oil red-O staining by optical density (OD absorption, 492nm). N=3 independent experiments, *p<0.05 50mM EtOH *versus* 0mM EtOH, $^{\#}$p<0.05 AS + 50mM EtOH *versus* 50mM EtOH.

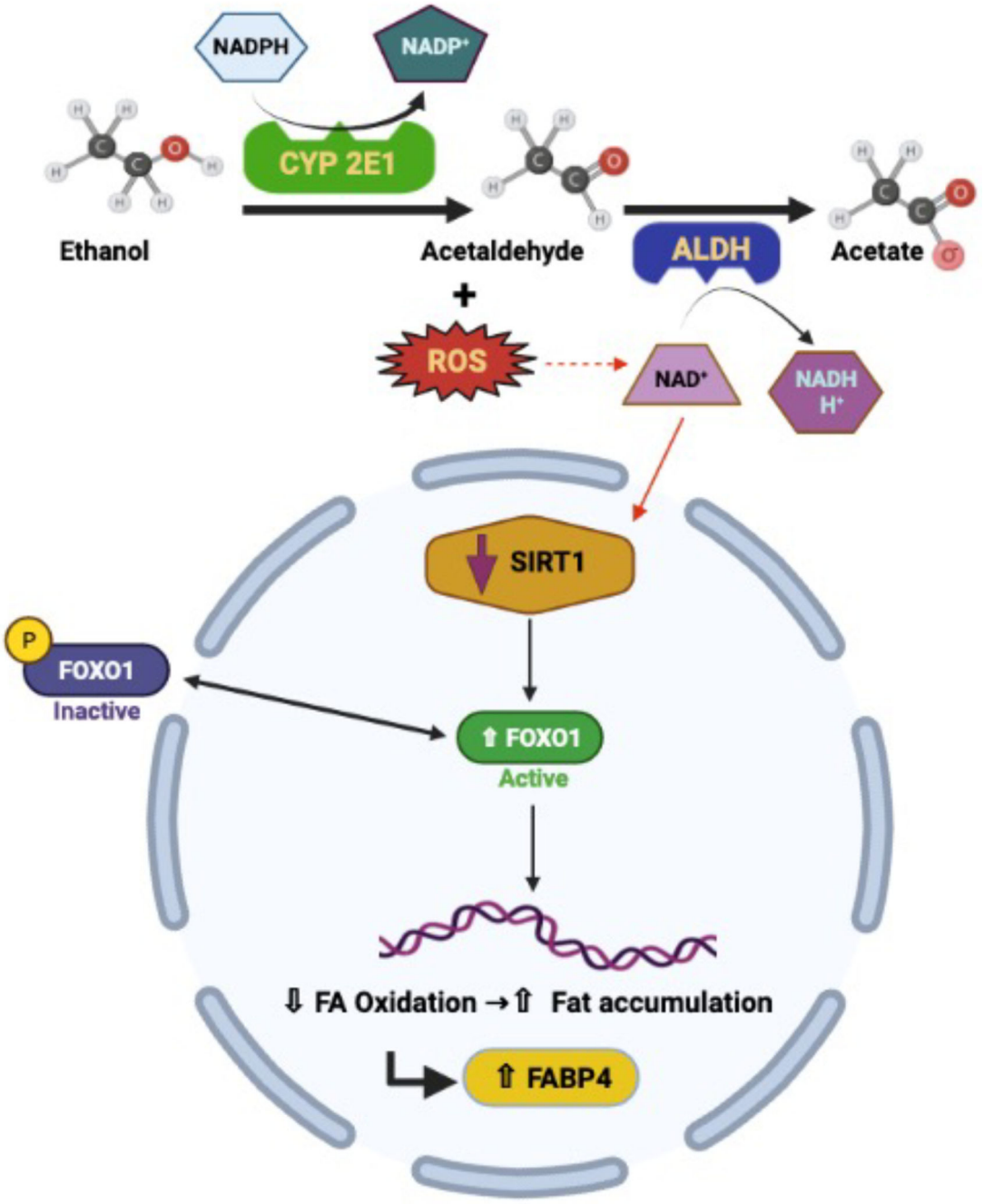

Figure 6. ***Potential mechanisms by which CYP2E1-catalyzed ethanol metabolism alters hepatic* FABP4 *mRNA expression and FABP4 protein synthesis.***

Chronic ethanol (EtOH) exposure induces CYP2E1 induction which utilizes NADPH as a cofactor to oxidize EtOH to acetaldehyde and generate reactive oxygen species (ROS). Increased acetaldehyde/ROS decreases sirtuin 1 (SIRT1) *via* decreased intracellular NAD^+ availability. Decreased SIRT1 leads to reduced pFOXO1 translocation to the cytoplasm and increased nuclear FOXO1 protein levels Elevated nuclear FOXO1 leads to decreased fatty acid β-oxidation/increased lipogenesis and intracellular neutral lipid accumulation resulting

in increased fatty acid binding protein-4 (*FABP4*) mRNA expression and FABP4 protein secretion.