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Role of ERK1/2 Signaling in Cinnabarinic Acid-Driven Stanniocalcin 2–Mediated Protection against Alcohol-Induced Apoptosis

Nikhil Y. Patil, Iulia Rus, and Aditya D. Joshi

Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

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ABSTRACT

We have previously shown that a bona fide aryl hydrocarbon receptor (AhR) agonist, cinnabarinic acid (CA), protects against alcohol-induced hepatocyte apoptosis via activation of a novel AhR target gene, stanniocalcin 2 (Stc2). Stc2 translates to a secreted disulfide-linked hormone, STC2, known to function in cell development, calcium and phosphate regulation, angiogenesis, and antiapoptosis—albeit the comprehensive mechanism by which the CA-AhR-STC2 axis confers antiapoptosis is yet to be characterized. In this study, using RNA interference library screening, downstream antiapoptotic molecular signaling components involved in CA-induced STC2-mediated protection against ethanol-induced apoptosis were investigated. RNA interference library screening of kinases and phosphatases in Hepa1 cells and subsequent pathway analysis identified mitogen-activated protein kinase (MAPK) signaling as a critical molecular pathway involved in CA-mediated protection. Specifically, phosphorylation of ERK1/2 was induced in response to CA treatment without alterations in p38 and JNK signaling pathways. Silencing Stc2 in Hepa1 cells and in vivo experiments performed in Stc2$^{-/-}$ (Stc2 knockout) mice, which failed to confer CA-mediated protection against ethanol-induced apoptosis, showed abrogation of ERK1/2 activation, underlining the significance of ERK1/2 signaling in CA-STC2–mediated protection. In conclusion, activation of ERK1/2 signaling in CA-driven AhR-dependent Stc2-mediated protection represents a novel mechanism of protection against acute alcohol-induced apoptosis.

SIGNIFICANCE STATEMENT

Previous studies have shown the role of stanniocalcin 2 (Stc2) in cinnabarinic acid (CA)-mediated protection against alcohol-induced apoptosis. Here, using RNA interference library screening and subsequent in vivo studies, the functional significance of ERK1/2 activation in CA-induced Stc2-mediated protection against acute ethanol-induced apoptosis was identified. This study is thus significant as it illustrates a comprehensive downstream mechanism by which CA-induced Stc2 protects against alcoholic liver disease.

Introduction

Binge alcohol intake is the most common form of excessive alcohol use, and repeated episodes of binge drinking lead to a spectrum of alcohol-related liver injuries, including alcoholic fatty liver (hepatic steatosis), alcoholic steatohepatitis (hepatic steatosis with inflammation), and alcoholic hepatitis (O'Shea et al., 2010; Seitz et al., 2018). In acute ethanol-induced liver injury, hepatocytes are damaged due to oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction, which manifests in the form of inflammation, apoptosis, necrosis, and metabolic changes (Ishii et al., 2003; Nagy et al., 2016; Subramaniyan et al., 2021). Several studies have investigated the link between hepatocyte apoptosis and alcohol-induced liver diseases, which demonstrated that, compared with a healthy liver, the rate of hepatocyte apoptosis is significantly higher in alcoholic liver diseases, and it correlates with the severity of the disease (Benedetti et al., 1988; Zhou et al., 2001, 2003; Ghosh Dastidar et al., 2018). Furthermore, preventing hepatocyte apoptosis has shown to alleviate liver injury (Wang et al., 2016; Zhu et al., 2017; Lee et al., 2019). Therefore, identifying the protective mechanisms against apoptosis can serve in developing therapeutic avenues targeting alcoholic liver diseases.

The aryl hydrocarbon receptor (AhR), known for its role in xenobiotic metabolism and hepatic homeostasis, is highly expressed in the liver (Mimura and Fujii-Kuriyama, 2003; Savouret et al., 2003; Wu et al., 2007). In its inactivated form, AhR resides in the cytosol, but ligand binding results in its translocation to nucleus, where AhR dimerizes with the AhR nuclear translocator and regulates transcription of various genes, including those involved in phase I and II xenobiotic metabolism, including cytochrome P450 1A1 (Cyp1a1) (Reyes et al., 1992; Probst et al., 1993; Wright et al., 2017). Apart from prototypical exogenous AhR ligands of natural and anthropic origin, recent studies have identified a plethora of endogenous agonists for AhR (Nguyen and Bradfield, 2008;

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; CA, cinnabarinic acid; ERK, extracellular signal-regulated kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; NT, nontargeting; RT-PCR, real-time polymerase chain reaction; Stc2, stanniocalcin 2.

Granados et al., 2022). Cinnabarinic acid (CA), a tryptophan metabolite of the kynurenine pathway, is a well characterized AhR agonist that does not activate classic AhR target gene Cyp1a1 but induces expression of a novel gene, stanniocalcin 2 (Stc2), in the liver (Harper et al., 2013; Joshi et al., 2015b). CA-inducible AhR target, Stc2 translates to a secreted disulfide-linked hormone, STC2, known to function in cell growth, development, antiapoptosis, calcium and phosphate regulation, and angiogenesis (Joshi, 2020). Our laboratory has previously reported that CA-induced AhR-dependent STC2 upregulation as well as direct STC2 overexpression by plasmid delivery protected isolated primary hepatocytes against apoptosis induced by ethanol (Joshi et al., 2015a). Furthermore, in vivo studies using mouse models of acute and chronic alcoholic liver disease showed CA-mediated protection against ethanol-induced apoptosis, hepatic steatosis, and liver injury (Joshi et al., 2015a, 2022). However, CA did not protect hepatocyte-specific AhR knockout mice livers against ethanol-induced apoptosis, establishing that the CA-driven hepatoprotection was dependent on hepatic AhR (Joshi et al., 2015a). Recently, we demonstrated that in $Stc2^{-/-}$ (Stc2 knockout) mice, CA failed to protect against alcoholic liver injury and apoptosis in both acute and chronic models, whereas reintroduction of Stc2 in the liver using in vivo gene delivery restored cytoprotection against injury (Joshi et al., 2022). Although these studies strongly indicate that CA-induced AhR-dependent protection against ethanol-induced apoptosis is contingent upon STC2 upregulation, the exact mechanism by which STC2 exerts antiapoptotic function has yet to be unraveled.

In the present study, we performed targeted high-throughput RNA interference library screening of kinases and phosphatases to identify antiapoptotic genes and downstream pathways involved in CA-induced AhR-STC2–mediated protection against acute ethanol-stress. The current study identified the significant role of the extracellular signal-regulated kinase (ERK)-1/2 pathway in CA-driven STC2-mediated protection against acute alcoholic liver disease.

Materials and Methods

Cell Culture and in Vitro Treatments. A murine hepatoma cell line, Hepa1 (Hepa1c1c7) cells were cultured in α minimum essential medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS (R&D Biosystems, Minneapolis, MN) and 1% 100× penicillin-streptomycin solution (ThermoFisher Scientific). Cells were maintained at 37°C in a humidified 5% CO_2 incubator. Cells were grown in six-well plates at 90% confluency and treated with DMSO for 24 hours (control), 30 μM CA dissolved in DMSO for 24 hours (CA), 100 mM ethanol for 24 hours (ethanol), and 30 μM CA for 24 hours followed by 100 mM ethanol for another 24 hours (ethanol plus CA). For the studies involving RNA interference, Hepa1 cells were transfected with 50 nM Dharmacon SMARTpool ON-TARGETplus AhR, Stc2, and nontargeting (NT, scrambled) siRNAs using Metafectene FluoR (Biontex, Munchen, Germany) transfection reagent according to the manufacturer's protocol. Twenty-four hours later, cells were treated with DMSO, 30 μM CA, and 100 mM ethanol for 24 hours and with 30 μM CA for 24 hours followed by 100 mM ethanol for another 24 hours. For ERK inhibition studies, Hepa1 cells were treated with DMSO (vehicle) or 30 μM CA or transfected with Stc2/pCMV-SPORT6 plasmid (Horizon Discovery) using Metafectene FluoR (Biontex, Munchen, Germany) for 24 hours, with or without 10 μM SCH772984 pretreatment of 2 hours. Except the control group, the remaining groups were treated with 100 mM ethanol for an additional 24 hours.

RNA Interference Library Screening and Pathway Analysis. The Dharmacon ON-TARGETplus siRNA library for mouse protein kinases and phosphatases was sourced from Horizon Discovery. The library was composed of a mixture of four different sequences of siRNAs (SMARTpool) targeting each of the 714 protein kinase and 272 phosphatase genes, arrayed in 96-well plates. The siRNA libraries were resuspended in Dharmacon 5× siRNA buffer (Horizon Discovery), according to the manufacturer's instructions, to make a stock concentration of 100 μM. Hepa1 cells of identical passage number were plated in an antibiotic-free medium in 96-well plates at a density of 10^4 cells/well. Cells were then transfected with gene-specific and NT (scrambled) siRNA at a final concentration of 50 nM for 24 hours using DharmaFECT 4 transfection reagent (Horizon Discovery) according to the manufacturer's protocol. The cells were further treated with 30 μM CA dissolved in DMSO for 24 hours followed by 100 mM ethanol for another 24 hours. To account for the background luminescence, each plate had a no-cell control consisting of growth medium only. A known apoptosis inducer, staurosporine (10 μM), was used as a positive control for each plate. Data were analyzed by subtracting luminescence values from the growth-media-only control well from all other wells. Next, luminescence values of the silenced (specific kinases and phosphatases) and treated samples (siRNA plus CA plus ethanol) were normalized to the values of the nonsilenced (nontargeting/scrambled siRNA control) and treated samples (NT-siRNA plus CA plus ethanol). Genes that, when silenced, showed higher apoptosis than NT-siRNA plus CA plus ethanol samples were considered putative targets and were subjected to pathway analysis tools. The pathway enrichment analysis to decipher comprehensive and objective global network, including direct and indirect interactions, was performed using STRING, which implements well known, curated pathway databases, including Gene Ontology, Reactome, and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Szklarczyk et al., 2021).

Animals and in Vivo Treatments. Eight- to 10-week-old wild-type ($Stc2^{+/+}$) and Stc2 knockout ($Stc2^{-/-}$) female mice (Zeiger et al., 2011; Joshi et al., 2022) were used in compliance with the guidelines of the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center. The mice were given an acute alcohol treatment of 6 g/kg (body weight) ethanol for 12 hours through oral gavage. The mice in the ethanol plus CA group were pretreated with 12 mg/kg CA for 12 hours via intraperitoneal injections, following which ethanol was administered for another 12 hours. Twelve hours following the ethanol treatment, mice were euthanized, and liver tissue was harvested. This model of acute alcohol-induced apoptosis has been previously well characterized (Zhou et al., 2001, 2003; Joshi et al., 2015a, 2022).

Histopathology. Livers from control, CA, ethanol, and ethanol plus CA treatment groups were fixed with 10% neutral buffered formalin solution and submitted to the Histology Core Laboratory at the University of Oklahoma Health Sciences Center for paraffin embedding, sectioning, and H&E staining.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction. Cells were rinsed with PBS and total RNA was extracted using TRIzol reagent (Thermofisher Scientific) and quantified. First-strand cDNA was synthesized from 1 μg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using the PowerUp SYBR Green Master Mix (ThermoFisher Scientific) and oligonucleotide primers for mouse Stc2 (forward 5′-GTCGGTGTGA TTGTGGAGATGAT-3′, reverse 5′-TCCACATAGGGCTCATGCAG-3′) on a StepOnePlus real-time PCR system (ThermoFisher Scientific). Expression of each gene was normalized to mouse 18S ribosomal RNA (forward 5′-CTCAACACGGGAAACCTCAC-3′, reverse 5′-CGCTCCAC-CAACTAAGAACG-3′).

Caspase-3/7 Apoptosis Assay. Hepa1 cell lysates and liver tissue homogenates were subjected to apoptosis assays as previously described (Joshi et al., 2022). Apoptosis was measured using the ApoTox-Glo Triplex Assay (Promega, Madison, WI) following the manufacturer's instructions. In brief, for each well of the 96-well plate, 100 μl of Caspase-Glo 3/7 reagent was added post-treatment and incubated for 1 hour at room temperature. The luminescence generated by the cleavage of DEVD-peptide

substrate by caspase 3/7 activity was recorded as a measure of apoptotic cell death on the Synergy 2 plate reader (Agilent, Santa Clara, CA).

Western Blot Analysis. Hepa1 cell lysates and liver homogenates were subjected to SDS-PAGE (Bio-Rad), followed by transfer to low-fluorescence polyvinylidene difluoride (PVDF) membranes. The membranes were probed with antibodies against AhR (Enzo Lifesciences, Farmingdale, NY), Stc2 (ProSci Inc, Poway, CA), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), p38, phospho-p38 (Thr180/Tyr182), JNK, phospho-JNK (Thr183/Tyr185), Akt, phospho-Akt (Ser473) (Cell Signaling Technology, Inc., Danvers, MA), and actin (Sigma-Aldrich, Inc., St. Louis, MO). Proteins were detected using the fluorescently labeled secondary antibodies goat anti-rabbit StarBright 700 and goat anti-mouse StarBright 520 (Bio-Rad) and imaged on the ChemiDoc MP imaging system (Bio-Rad).

Statistical Analysis. Acute alcohol treatment on cells and animal studies were conducted with a predetermined plan. Animal and cell/sample sizes per group, data acquisition, and analysis methodologies were also preset (Michel et al., 2020). Animal sizes were determined by calculating effect size based on our previous acute-alcohol study and by performing power analysis calculations using G*Power statistical suite 3.1 with type I error set at 5% and type II error at 80% (Faul et al., 2009; Joshi et al., 2022). For the comparison of two groups, Student's t test was performed. For multiple groups, ANOVA with post hoc Tukey's procedure was conducted using the Sigma Plot software (Systat software, San Jose, CA). All data unless specified are represented as mean ± S.D. Differences between the groups are considered statistically significant if $P < 0.05$.

Results

CA-Induced AhR-Regulated Stc2 Protects against Ethanol-Induced Apoptosis in Hepa1 Cells. Our laboratory has previously demonstrated that an endogenous AhR agonist, cinnabarinic acid, upregulates hepatic expression of a novel AhR target gene, Stc2 (Joshi et al., 2015a, 2022). Furthermore, in isolated mouse primary hepatocytes and in vivo, CA was able to protect against alcohol-induced apoptosis, and the protection was both AhR and STC2 dependent (Joshi et al., 2015a, 2022). To identify genes/pathways downstream of CA-induced AhR-dependent STC2 involved in protection against ethanol-induced hepatic apoptosis, we used RNA interference library screening in Hepa1 cells. First, CA-mediated induction of Stc2 mRNA and subsequent protection against ethanol-induced apoptosis in Hepa1 cell line was validated. Using quantitative RT-PCR, we confirmed approximately fivefold upregulation of Stc2 mRNA message upon 30 μM CA treatment compared with the control (DMSO) (Fig. 1A). We then assessed the potential of CA to protect Hepa1 cells against ethanol-induced apoptosis using caspase 3/7 activity assay. As expected, ethanol treatment induced (approximately fourfold) programmed cell death compared with the control-only group. Pretreatment with CA significantly reduced apoptosis induced by ethanol stress and conferred protection (Fig. 1B). Next, we validated the role of AhR and Stc2 in CA-mediated protection against ethanol-induced apoptosis in Hepa1 cells. Hepa1 cells were transiently transfected with gene-specific (AhR, Stc2) and NT (scrambled) siRNA oligonucleotides followed by CA and ethanol treatments. Suppression of AhR and Stc2 expression was confirmed by western blotting (Fig. 1C). In AhR- and Stc2-positive cells(NT siRNA treated), CA was able to alleviate ethanol-induced apoptosis. On the contrary, CA failed to protect against apoptosis when either AhR or

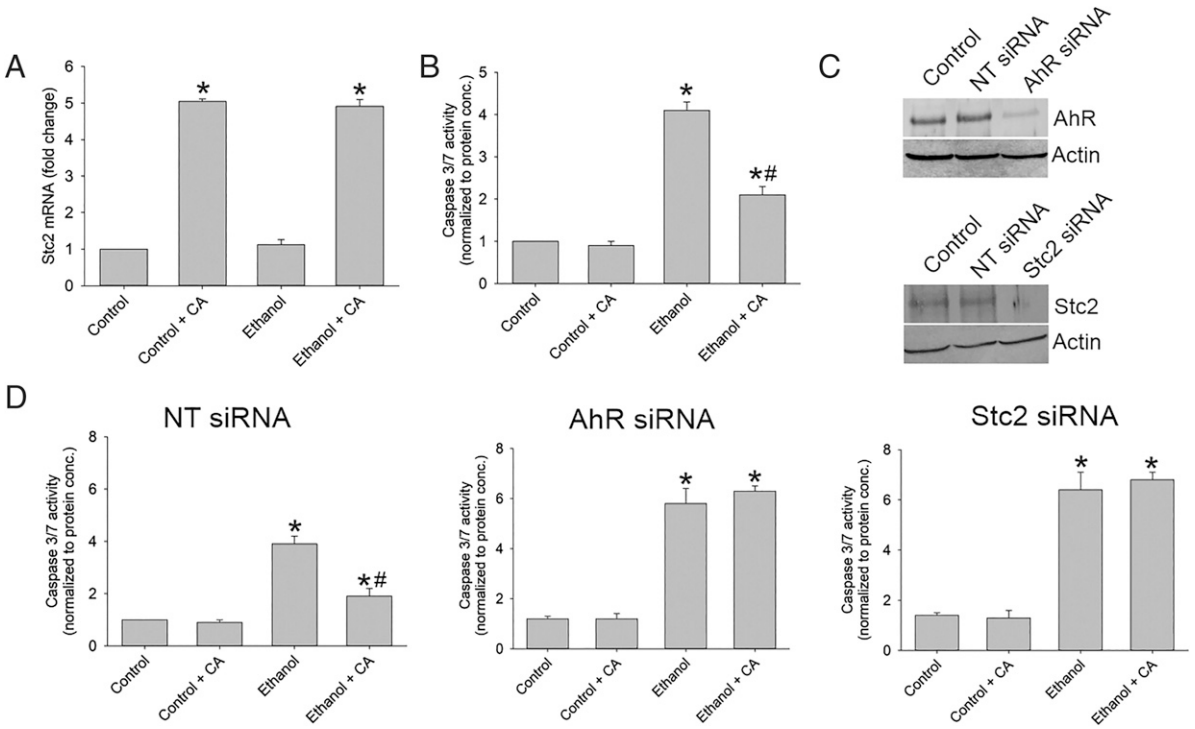

Fig. 1. CA upregulates Stc2 expression and protects against acute ethanol-induced apoptosis in an AhR- and Stc2-dependent manner. Hepa1 Cells were treated with DMSO (control), 30 μM CA dissolved in DMSO for 24 hours (control plus CA), 100 mM ethanol for 24 hours (ethanol), and 30 μM CA for 24 hours followed by 100 mM ethanol for another 24 hours (ethanol plus CA). (A) Stc2 mRNA was measured by quantitative RT-PCR and normalized against 18S ribosomal RNA. Results are expressed as fold value compared with the control treatment. (B) Caspase 3/7 activity was recorded as a measure of apoptotic cell death and normalized to total protein concentration and to control treatment. Hepa1 cells were transiently transfected with AhR, Stc2, and NT siRNA for 24 hours. (C) Western blotting was performed to confirm knockdown of AhR and Stc2, and (D) apoptosis was measured 24 hours after CA (30 μM) and/or ethanol (100 mM) treatments of the cells. Data are represented as mean ± S.D. ($n = 3$). *$P < 0.05$ compared with control treatment; #$P < 0.05$ compared with ethanol-only treatment.

Stc2 was knocked down (Fig. 1D). Moreover, in the absence of CA treatment, increased caspase 3 activity was observed in ethanol-only groups when AhR and Stc2 were silenced, suggesting an intrinsic cytoprotective role of both AhR and Stc2 (Fig. 1D). These data are in congruence with our previous studies performed in isolated mouse primary hepatocytes and in vivo (Joshi et al., 2015a, 2022) and confirm that the antiapoptotic effect exerted by CA against ethanol-induced toxicity are dependent on AhR-mediated Stc2 signaling in Hepa1 cells.

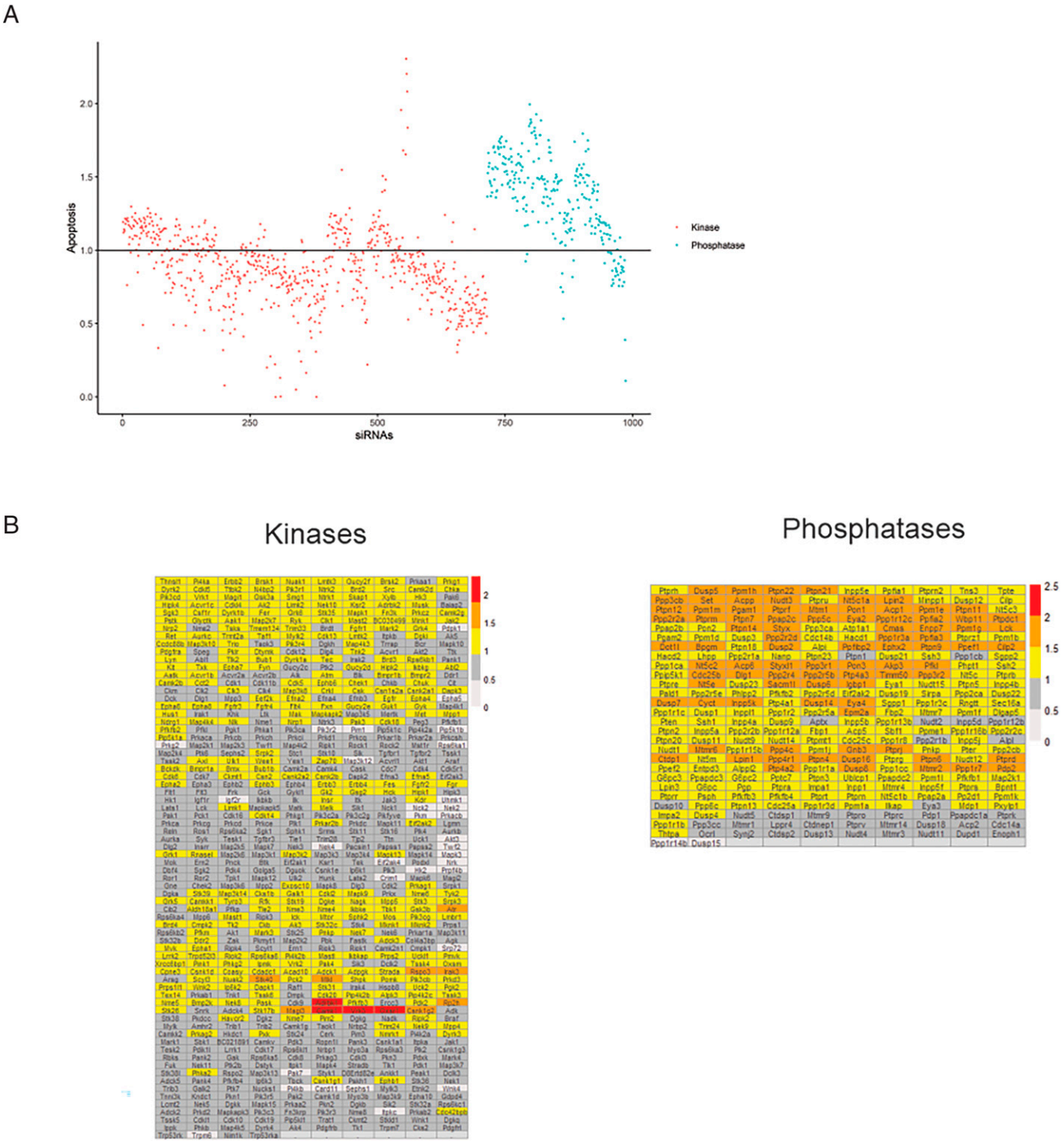

Fig. 2. RNA interference library screening identified critical protein phosphatases and kinases involved in CA-driven protection against ethanol-induced apoptosis. Hepa 1 cells were plated in 96-well plates, transfected with gene-specific siRNAs or NT-siRNA pool (nontargeting/scrambled) for 24 hours, and treated with 30 μM CA (for 24 hours) and 100 mM ethanol for additional 24 hours. The apoptosis signal from kinase- and phosphatase-specific gene knockdown wells was measured using the ApoTox-Glo triplex assay and normalized to the signal from the NT-siRNA plus CA plus ethanol samples (set at 1) and graphically displayed as (A) dot plot (orange, kinases; blue, phosphatases) and (B) heatmap.

A

KEGG

Pathway	Description
mmu04010	MAPK signaling pathway
mmu00562	Inositol phosphate metabolism
mmu0110	Metabolic pathways
mmu04070	Phosphatidylinositol signaling
mmu04152	AMPK signaling pathway
mmu04151	PI3K-Akt signaling pathway
mmu04910	Insulin signaling pathway
mmu04360	Axon guidance
mmu04660	T cell receptor signaling pathway
mmu04071	Sphingolipid signaling pathway

B

GO

Pathway	Description
GO:0000165	MAPK cascade
GO:0000186	Activation of MAPKK activity
GO:0000187	Activation of MAPK activity
GO:0000188	Inactivation of MAPK activity
GO:0000278	Mitotic cell cycle
GO:0000904	Cell morphogenesis involved in differentiation
GO:0000902	Cell morphogenesis
GO:0000302	Response to reactive oxygen species
GO:0000086	G2M transition of mitotic cell cycle
GO:0001558	Regulation of cell growth

C

Reactome

Pathway	Description
MMU-1430728	Metabolism
MMU-5684996	MAPK1/MAPK3 signaling
MMU-5683057	MAPK family signaling cascade
MMU-1483255	PI metabolism
MMU-5673001	RAF/MAP kinase cascade
MMU-5675221	Negative regulation of MAPK pathway
MMU-9006934	Signaling by receptor tyrosine kinase
MMU-1483257	Phospholipid metabolism
MMU-199418	Negative regulation of the PI3K/Akt network
MMU-162582	Signal transcduction

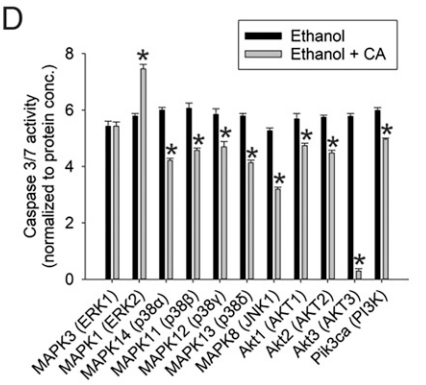

Fig. 3. Pathway enrichment analysis identified the association of MAPK and AKT signaling pathways with CA-mediated protection against ethanol-induced apoptosis. Top enriched terms identified by well known classification systems (A) KEGG, (B) Gene Ontology, and (C) Reactome are depicted. The term at the top has the most significant overlap with the input query gene set. (D) For a secondary screening, Hepa1 cells were plated in a 96-well plate, transfected with siRNAs targeting members of MAPK (ERK1/2, p38$\alpha/\beta/\gamma/\delta$, JNK1) and AKT (Akt1/2/3, PI3K) pathways for 24 hours, and treated with 30 μM CA for 24 hours and 100 mM ethanol for an additional 24 hours. Caspase 3/7 activity was measured. Data are represented as mean ± S.D. ($n = 3$). *$P < 0.05$ compared with ethanol-only treatment.

High-Throughput RNA Interference Library Screening Identified Critical Genes Involved in CA-Mediated Protection. We next sought to identify signal transduction genes involved downstream of CA-induced STC2-mediated cytoprotective cascade. Since protein kinases and phosphatases are known to play a prominent role in modulating pathways in apoptosis signaling, we employed a high-throughput RNA interference library screening to identify key kinases and phosphatases involved in CA-AhR-STC2–mediated protection against ethanol-induced apoptosis. The library comprised a set of four siRNAs per target gene to maintain high-silencing potency and modification patterns to reduce off-target effects. The apoptotic signal from each well of the library transfected with gene-specific siRNA pool and treated with 30 μM CA and 100 mM ethanol was measured and normalized to the signal from the NT-siRNA plus 30 μM CA plus 100 mM ethanol samples. A total of 214 kinases and 235 phosphatases, when silenced, showed increased ethanol-induced apoptosis compared with the nonsilenced group in spite of CA treatment. These 449 kinase and phosphatase genes, which, when knocked down, failed to protect cells from ethanol-induced apoptosis despite having an activated CA-induced AhR-dependent STC2 protection, were considered to be involved in the downstream antiapoptotic pathway and were subjected to pathway enrichment analysis (Fig. 2, A and B).

CA-Mediated Protection against Ethanol-Induced Apoptosis Involves Mitogen-Activated Protein Kinase Signaling Pathway. A pathway enrichment analysis was performed on the kinases and phosphatases that are involved in modulating CA-driven protection against ethanol-induced apoptosis (Joshi et al., 2015a, 2022). The input gene names were converted to corresponding protein names and analyzed against the comprehensive databases of cell signaling and metabolic pathways. The Gene Ontology, KEGG, and Reactome pathway libraries identified multiple biologic pathways regulated in response to CA treatment. Enriched pathways were screened, and top pathways were identified based on the total counts of genes/proteins in the network and false discovery rate (Fig. 3, A–C). The PI3K-Akt pathway was identified by both Reactome and KEGG, whereas MAPK signaling was detected by all three pathway enrichment analysis tools (Fig. 3, A–C). Since both Akt and mitogen-activated protein kinase (MAPK) pathways have been implicated to play critical role in alcohol-induced apoptosis (Aroor and Shukla, 2004; Han et al., 2020), we validated the involvement of these pathways in CA-driven protection. For a secondary screening, genes encoding members of Akt and three components of MAPK pathway, namely ERK1/2, p38, and JNK, were knocked down in Hepa1 cells using RNA interference. Silencing p38 (encoded by p38$\alpha/\beta/\gamma/\delta$), JNK1 (MAPK8), and Akt-PI3K (Akt1/2/3 and Pik3ca) had no effect on CA-mediated protection against ethanol-

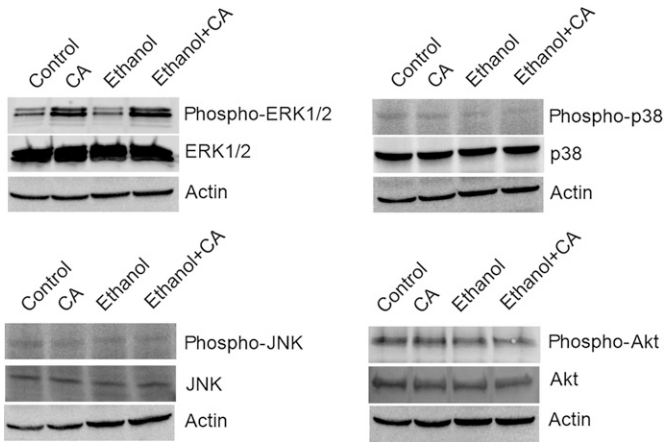

Fig. 4. CA activates the ERK1/2 pathway in vitro. Western blotting was performed on Hepa1 cells treated with DMSO (control), 30 μM CA (CA), 100 mM ethanol (ethanol) for 24 hours, or 30 μM CA for 24 hours followed by 100 mM ethanol for an additional 24 hours (ethanol plus CA) to monitor expression of total and phosphorylated ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), JNK (Thr183/Tyr185), and Akt (Ser473). A representative blot is shown out of three independent experiments.

induced apoptosis, implying minimal contribution of these pathways in hepatoprotection. However, CA failed to protect against apoptosis when ERK1/2 were silenced (Fig. 3D). This indicates the potential involvement of the ERK1/2 pathway in CA-driven protection against alcohol-induced apoptosis.

CA Activates the ERK1/2 Pathway. Next, the functional significance of the MAPK pathway in CA-mediated protection was investigated. Western blotting indicated increased phosphorylation of ERK1/2 (Thr202/Tyr204) in response to CA treatment compared with control and ethanol-only groups (Fig. 4). In contrast, the phosphorylation status of p38 (Thr180/Tyr182) and JNK (Thr183/Tyr185) was not different in control or any of the treatment groups. CA treatment was not able to regulate expression of phospho-Akt (Ser473) (Fig. 4). To further investigate whether CA exerted its cytoprotective function via activation of the ERK1/2 pathway in vivo, we used an acute model of alcohol-induced liver injury. This well established single-binge model of in vivo acute ethanol treatment is known to induce hepatocyte apoptosis and liver injury with microvesicular steatosis (Zhou et al., 2001, 2003; Joshi et al., 2015a, 2022; Ghosh Dastidar et al., 2018). Wild-type mice were treated with a single dose of 6 g/kg (body weight) ethanol orally for 12 hours in the absence (saline, control) and presence of 12 mg/kg CA pretreatment as previously described (Joshi et al., 2015a, 2022). An acute ethanol treatment elevated hepatocyte apoptosis as measured using caspase 3/7 assays (Fig. 5A) and resulted in a microvesicular steatosis (Fig. 5B). CA administration was able to protect against ethanol-induced apoptosis (Fig. 5A) and steatosis (Fig. 5B), which corroborated with our previous studies (Joshi et al., 2022). Immunoblotting performed on livers indicated activated ERK1/2 signaling in response to CA treatment, whereas CA had no effect on phosphorylation of p38 and JNK (Fig. 5C). The cumulative data thus indicate that CA activate hepatic ERK1/2 signaling both in vitro and in vivo and protect against ethanol-induced apoptosis.

CA-Induced Stc2 Expression Is Critical for the Regulation of ERK1/2 and Mitigation of Apoptosis. We next evaluated whether CA-induced ERK1/2 activation and protection against apoptosis is Stc2 dependent. In the presence of CA, Hepa1 cells transfected with siRNA to knock down Stc2 did not activate ERK1/2 phosphorylation (Fig. 6A) and failed to protect against ethanol-induced apoptosis (Fig. 1D). In an in vivo study, a single binge of ethanol elevated hepatic apoptosis in $Stc2^{-/-}$ (Stc2 knockout) mice livers compared with $Stc2^{+/+}$ (wild type) (Figs. 5A and 6B). CA treatment neither attenuated ethanol-induced cell death (Fig. 6B) nor mitigated hepatosteatosis (Fig. 6C), thus validating the in vivo role of Stc2 in CA-mediated protection (Joshi et al., 2022). In $Stc2^{-/-}$

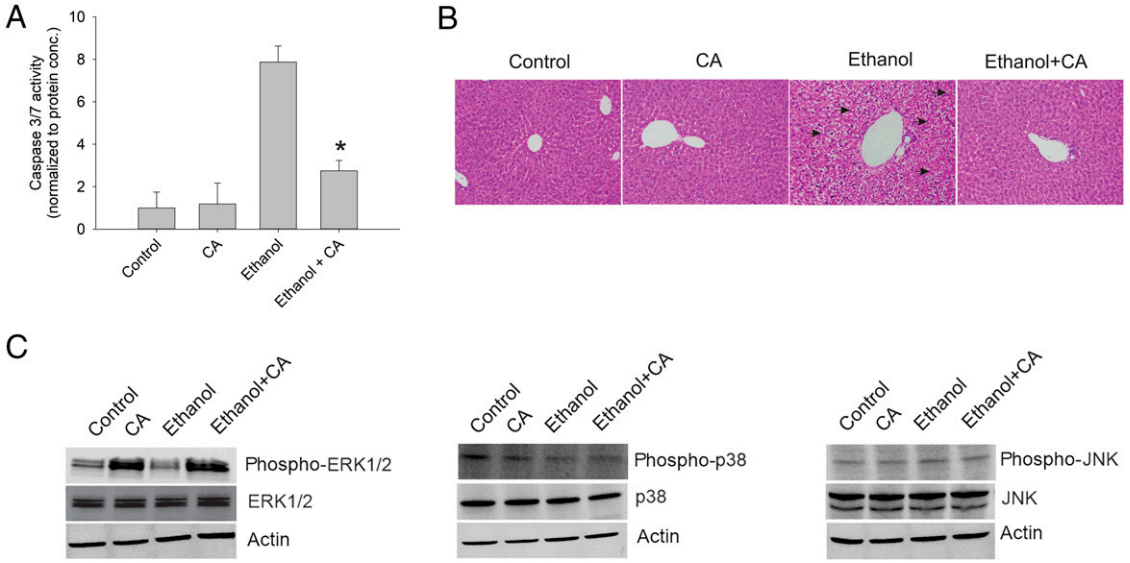

Fig. 5. CA phosphorylates ERK1/2 and protects against acute ethanol-induced apoptosis in vivo. Wild-type mice were treated with saline (control), 12 mg/kg (body weight) CA (CA group) for 24 hours, 6 gm/kg (body weight) ethanol (Ethanol group) for 12 hours, or with CA for 12 hours prior to ethanol for 12 hours (ethanol plus CA group). (A) Liver homogenates were subjected to caspase 3/7 activity assay. The results are shown as the mean ± S.D. from four independent experiments. Data are normalized to the caspase 3/7 activity in the control group. *$P < 0.05$ represents a statistically significant difference between ethanol-treated and ethanol plus CA-treated groups. (B) H&E staining depicting histopathological changes in the liver. Arrows indicate microvesicular steatosis. (C) Immunoblotting was performed on liver lysates to monitor expression of phosphorylated and total ERK1/2, p38, and JNK. Actin was used as a control. A representative blot out of four independent blots is shown.

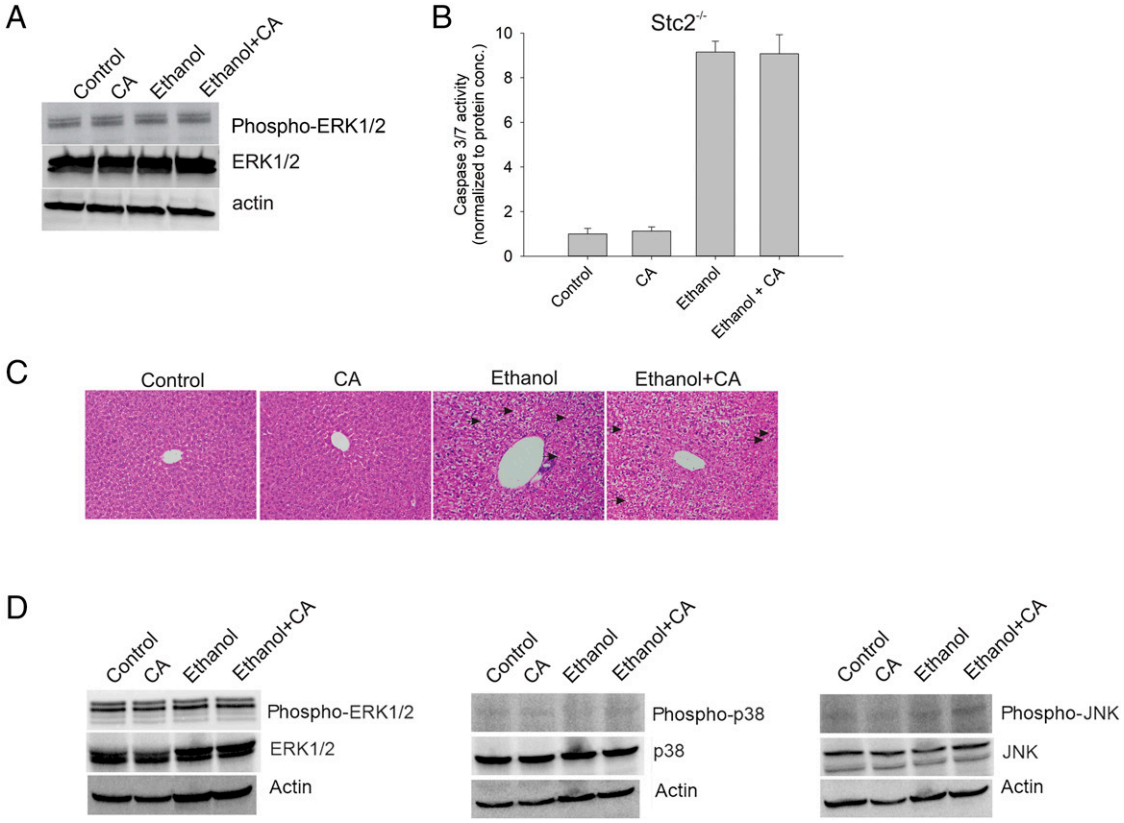

Fig. 6. CA-mediated activation of ERK1/2 is Stc2 dependent. (A) Hepa1 cells were transiently transfected with Stc2 siRNA for 24 hours and treated with DMSO (control), 30 μM CA (CA), 100 mM ethanol (ethanol) for additional 24 hours, or 30 μM CA for 24 hours followed by 100 mM ethanol for 24 hours (ethanol plus CA). Western blotting was performed using actin as a loading control. A representative immunoblot is shown. (B) Caspase 3/7 activity assay was performed on liver homogenates from $Stc2^{-/-}$ (Stc2 knockout) mice and treated with saline (control), 12 mg/kg (body weight) CA (CA group) for 24 hours, 6 g/kg (body weight) ethanol (ethanol group) for 12 hours, or 12 mg/kg CA for 12 hours followed by 6 g/kg ethanol for an additional 12 hours (ethanol plus CA group). Data are normalized to the caspase 3/7 activity in the control group ($n = 4$). (C) H&E staining indicating microvesicular steatosis (arrow) and liver pathology. (D) Immunoblotting was performed on liver lysates with actin used as a loading control. A representative immunoblot is shown out of four independent experiments.

mice, CA treatment did not induce hepatic ERK1/2 phosphorylation (Fig. 6D), strongly suggesting the essential role of Stc2 in CA-induced ERK1/2 activation and antiapoptosis. CA and ethanol treatment had no effect on the phosphorylation of p38 and JNK in $Stc2^{-/-}$ livers (Fig. 6D).

To underline the role of ERK1/2 in CA-induced Stc2-mediated antiapoptosis, we treated Hepa1 cells with an ERK1/2 inhibitor, SCH772984. Stc2 expression upon CA treatment and due to recombinant plasmid delivery (Stc2/pCMV-SPORT6) was monitored by immunoblotting (Fig. 7A). Western blotting also confirmed inhibition of ERK1/2 phosphorylation upon SCH772984 treatment (Fig. 7A). Caspase 3/7 assays indicated that both CA-induced Stc2 upregulation and overexpression of a recombinant Stc2 protein induced ERK1/2 phosphorylation and protected Hepa1 cells against ethanol-induced apoptosis (Fig. 7B), whereas Stc2 did not confer protection against alcohol-induced apoptosis when ERK1/2 signaling was obliterated (Fig. 7B). Collectively, this study demonstrates the significant role of ERK1/2 activation in CA-driven STC2-mediated protection against acute alcohol-induced apoptosis.

Discussion

Alcohol abuse is a major health risk worldwide, leading to approximately 3.3 million deaths each year (Sudhinaraset et al., 2016). Binge drinking is the most common pattern of excessive alcohol use in the United States, involving one in six adults (CDC, 2012). The liver, being the major organ metabolizing alcohol, is at the forefront of the harm inflicted by alcohol intake, which leads to the manifestation of a broad range of liver diseases, from simple steatosis to cirrhosis and hepatocellular carcinoma (O'Shea et al., 2010; Seitz et al., 2018). Most forms of liver diseases are a direct or an indirect result of injury to the hepatocytes, which comprise 70%–80% of the liver cytoplasmic mass (Blouin et al., 1977). The initiation and/or progression of alcoholic liver disease is often characterized by marked stimulation of hepatocyte apoptosis (Zhao et al., 1997; Day, 2001; Feldstein and Gores, 2005). We have previously identified the ability of CA to protect against hepatocyte apoptosis in alcohol-induced liver diseases (Joshi et al., 2015a). This study thus exploits the formerly established CA-AhR-STC2–mediated hepatoprotective pathway to dissect downstream signaling cascades involved in protection against ethanol-induced apoptosis (Joshi et al., 2015a, 2022).

Prior studies by our group have demonstrated that the CA treatment protects against apoptosis, steatosis, and liver injury in chronic as well as acute models of ethanol feeding and promotes hepatocyte survival. Moreover, using hepatocyte-specific AhR knockout and Stc2 knockout mice livers, we showed the role of AhR-dependent STC2 signaling in CA-mediated antiapoptosis (Joshi et al., 2015a, 2022). To identify antiapoptotic

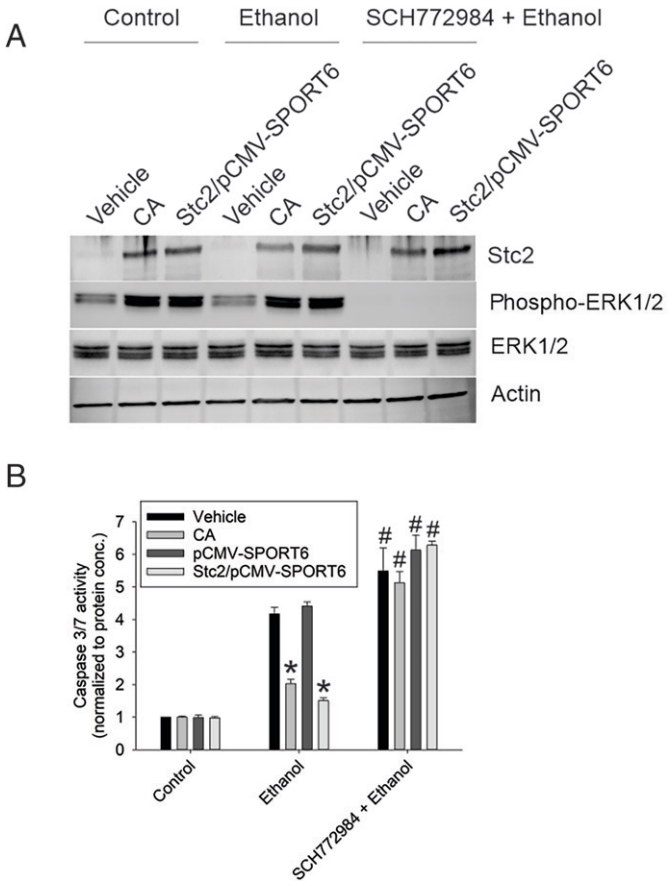

Fig. 7. ERK1/2 activation is critical for Stc2-mediated antiapoptosis. Hepa1 cells were treated with DMSO (vehicle), 30 μM CA (CA), transfected with a control vector (pCMV-SPORT6) or with a vector encoding recombinant Stc2 (Stc2/pCMV-SPORT6) for 24 hours. Cells within SCH772984 plus ethanol group were treated with 10 μM SCH772984, two hours before CA treatment or transfections. Twenty-four hours after CA treatment or transfections, cells in ethanol and SCH772984 plus ethanol groups were treated with 100 mM ethanol for an additional 24 hours. (A) Western blotting to monitor expression of Stc2, phospho-ERK1/2, and total ERK1/2. Actin was used as a loading control. A representative immunoblot is shown out of three independent experiments. (B) Cell lysates were subjected to caspase 3/7 assay to measure apoptotic cell death and normalized to total protein concentration and to the vehicle-treated control (no ethanol) group. The results are shown as the mean ± S.D. from three independent experiments. *$P < 0.05$ represents a statistically significant difference between ethanol-treated and ethanol plus CA–treated or between empty pCMV-SPORT6 and Stc2/pCMV-SPORT6 transfected groups. #$P < 0.05$ compared with ethanol-only treatment.

signaling pathways downstream of the CA-AhR-STC2 axis, we used a well characterized and widely used hepatic cell line, Hepa1 (Bernhard et al., 1973; Hankinson, 1979; Hankinson et al., 1991). These cells have been extensively used to characterize AhR biology and pathways regulated in response to AhR signaling (Hankinson, 1979; Hankinson et al., 1991; Tian et al., 1999; Abiko et al., 2015). Furthermore, the Hepa1 cell line has been used to decipher mechanisms of ethanol-induced apoptosis and to investigate potential therapeutics against alcoholic liver disease (Ushida and Talalay, 2013; Byun and Lee, 2015; Lee et al., 2022). In vitro studies using Hepa1 cells corroborated our previous findings observed in isolated mouse primary hepatocytes and in vivo, which showed induction of Stc2 and attenuation of ethanol-induced apoptosis in response to CA treatment (Fig. 1, A and B). Moreover, the protection was incumbent upon activation of AhR and expression of its target gene, Stc2 (Fig. 1D). The antiapoptotic and cytoprotective role of STC2 has been established by multiple previous studies (Ito et al., 2004; Joshi et al., 2015a, 2022; Patil et al., 2022). We have observed STC2-mediated protection against various cell death stimuli, including ethanol, H_2O_2, thapsigargin, and lipotoxicity (Joshi et al., 2015a, 2022; Patil et al., 2022). Moreover, increased STC2 expression resulted in reduced damage to acinar cells during pancreatitis, potentially by the upregulation of protein kinase R–like endoplasmic reticulum kinase (PERK) and activating transcription factor 4 (ATF4) signaling (Fazio et al., 2011). In human adipose-derived mesenchymal stem cells and human mesenchymal stem cells isolated from umbilical cord blood, overexpression of STC2 increased cell viability and survival under sublethal oxidative conditions. STC2 overexpression resulted in upregulation of cell cycle regulator proteins like cyclin-dependent kinase 2 and 4 (CDK2 and 4), downregulation of cell cycle inhibitors p16 and p21, and activation of phosphoprotein kinase B (pAKT) (Kim et al., 2015). STC2 has also been involved in ameliorating hepatosteatosis by regulating the signal transducer and the activator of transcription 3 (STAT3) pathway. It is known that STC2 is a secreted protein prominently found in the endoplasmic reticulum and Golgi apparatus of hepatocytes and acts in an autocrine/paracrine manner (McCudden et al., 2002; Ito et al., 2004; Joshi et al., 2015a; Sarapio et al., 2019). Confocal imaging of liver sections has identified the presence of STC2 immunostaining on plasma membrane in the form of punctate, indicating its potential interaction with a yet uncharacterized membrane receptor (Takei et al., 2009; Joshi et al., 2015a). Additionally, the downstream prosurvival signaling pathways activated by STC2 specifically in response to ethanol insult have not been comprehensively determined.

Protein phosphorylation is the most common post-translational modification that controls various aspects of cell fate, including metabolism, subcellular trafficking, inflammation, and apoptosis, by altering protein localization, stability, and/or enzymatic activity (Niemi and MacKeigan, 2013). To identify CA-driven STC2-mediated antiapoptotic pathways against ethanol-induced hepatotoxicity, we relied on the phospho-signaling network involving protein kinases and phosphatases, which regulate cell survival and apoptosis signals (Ardito et al., 2017). An RNA interference library screening was performed by individually silencing 986 kinases and phosphatases. The screening identified critical kinases and phosphatases, which, when knocked down, resulted in increased ethanol-induced cell death despite having an activated CA-induced STC2-mediated mechanism of cytoprotection (Fig. 2). A subsequent enrichment analysis identified multiple pathways, including Akt and MAPK signaling, potentially involved in CA-mediated protection. Akt is known to inhibit cytochrome c–induced caspase 9 activation, thus protecting against apoptosis (Zhou et al., 2000), and has been previously implicated in STC2-mediated protection (Kim et al., 2015). However, immunoblotting studies indicated that the CA treatment had no effect on phosphorylation of Akt, thus potentially marginalizing the contribution of the Akt pathway in CA-mediated antiapoptosis against acute ethanol stress (Fig. 4).

There is substantial evidence that activation of MAPK signaling and its effect in apoptosis in response to ethanol treatment is dictated by tissue/cell type as well as time and dosage regimen of ethanol (Aroor and Shukla, 2004). Upon ethanol treatment, we did not observe changes in basal ERK1/2, p38, and JNK phosphorylation in Hepa1 cells and liver tissues (Figs. 4 and 5). These observations correlate well with the

previous findings where the phosphorylation levels of ERK1/2 and JNK were not significantly different between the control and 24-hour ethanol-treated hepatocytes (Lee et al., 2002; Lee and Shukla, 2005; Gao et al., 2017). However, treatment with CA was able to induce one of the components of MAPK signaling, ERK1/2, both in vitro and in vivo (Figs. 4 and 5). In the absence of Stc2, CA failed to upregulate ERK1/2 phosphorylation and did not confer protection against ethanol-induced apoptosis (Figs. 1D and 6). Similarly, upon ablation of ERK activity, Stc2 did not protect against apoptosis (Fig. 7). This study thus underlines the importance of ERK1/2 activation in CA-induced, AhR-dependent, STC2-mediated protection against acute alcohol-induced apoptosis (Fig. 8). ERK1/2 activation has shown antiapoptotic effects in response to extrinsic as well as intrinsic stimuli through downregulation of proapoptotic and/or upregulation of antiapoptotic proteins (Lu and Xu, 2006). Several studies have indicated the prosurvival role of ERK1/2 activation in response to ethanol stress. In isolated rat hepatocytes, ERK1/2 activation conferred protection against ethanol-induced toxicity (Lee and Shukla, 2005). A downstream substrate of ERK1/2, RSK has shown to phosphorylate BAD and exert antiapoptotic effects (Shimamura et al., 2000). Moreover, studies have demonstrated that the treatment with quercetin and licochalcone B activates ERK1/2 signaling and protects against ethanol insult (Yao et al., 2007; Gao et al., 2017). Future studies, beyond the scope of this manuscript, will focus on characterizing the precise involvement of ERK1 and/or ERK2 signaling in CA-induced STC2-mediated hepatoprotection.

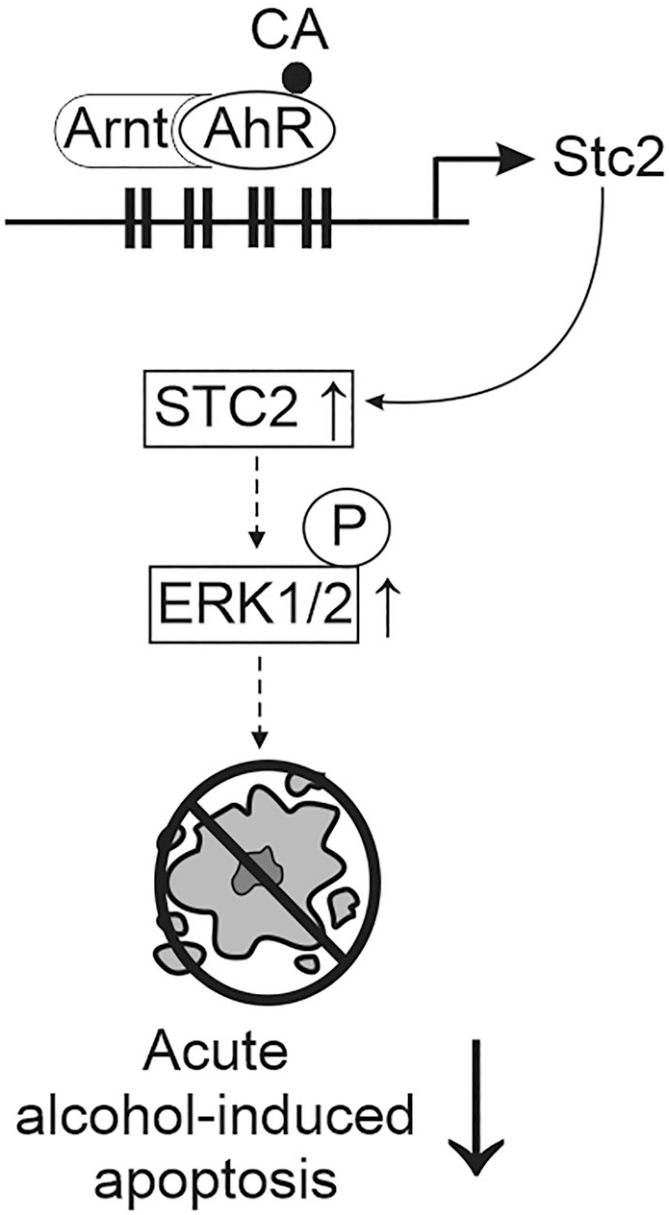

Fig. 8. An illustration depicting the role of ERK1/2 signaling in CA-induced, AhR-dependent, Stc2-mediated protection against acute ethanol-induced apoptosis. Upon endogenous AhR ligand CA binding, AhR translocate to the nucleus and interact to the xenobiotic response elements (depicted in black rectangles) present within the promoter of the Stc2 gene and elevate its expression. Upregulated STC2 induces the ERK1/2 signaling pathway and confers protection against alcohol-induced hepatocyte apoptosis.

The present study validated CA-mediated protection against ethanol-induced apoptosis by upregulation of AhR-dependent Stc2 in an in vitro model. Moreover, CA-induced Stc2 expression facilitated activation of the ERK1/2 signaling pathway and attenuated hepatic apoptosis in response to ethanol insult both in vitro and in vivo. In summary, this study has characterized the mechanism of hepatoprotection by CA-AhR-STC2 signaling and identified a significant role of the ERK1/2 pathway against acute alcohol-induced apoptosis.

Data Availability

The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions

Participated in research design: Patil, Joshi.
Conducted experiments: Patil, Rus.
Contributed new reagents or analytic tools: Patil, Rus, Joshi.
Performed data analysis: Patil, Joshi.
Wrote or contributed to the writing of the manuscript: Patil, Rus, Joshi.

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Address correspondence to: Dr. Aditya D. Joshi, Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave., Oklahoma City, Oklahoma 73117. E-mail: aditya-joshi@ouhsc.edu