Shc is implicated in calreticulin-mediated sterile inflammation in alcoholic hepatitis

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Short title: Shc and calreticulin in alcoholic hepatitis

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Abbreviations: 4-HNE, 4-hydroxynonenal; AAV, adeno-associated virus; ACAA2, 3-ketoacyl CoA thiolase; AH, alcoholic hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bak, Bcl-2 homologous antagonist killer; BAP31, B-cell receptor-associated protein 31; Bax, Bcl-2-associated X protein; CRT,
calreticulin; CXCL10, C-X-C chemokine ligand 10; DAMP, damage-associated molecular pattern; EIF2S1, eukaryotic transcription factor 2 subunit 1; ER, endoplasmic reticulum; IL-1 β, interleukin 1 beta; MCP1, monocyte chemoattractant protein 1; MCS-F, macrophage colony-stimulating factor; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; Shc, Src homology and collagen; Shc⁹⁰, Shc knockdown/Shc hypomorph; Shc⁹⁰HepKO, hepatocyte-specific Shc knockout; TG, triglyceride.

**Word Count:** 2348
Synopsis: We describe the role of Src homology and collagen proteins in alcoholic steatohepatitis. p46 Shc mediates lipid peroxidation, oxidative stress, and calreticulin translocation to the membrane where it acts as DAMP. Shc inhibition reduces inflammation in alcoholic liver injury.

Abstract

Background & Aims: Src homology and collagen (Shc) proteins are major adapters to extracellular signals however the regulatory role of Shc isoforms in sterile inflammatory responses in alcoholic hepatitis (AH) has not been fully investigated. We hypothesized that in an isoform-specific manner Shc modulates pre-apoptotic signals, calreticulin (CRT) membrane exposure and recruitment of inflammatory cells.

Methods: Liver biopsy samples from patients with AH vs. healthy subjects were studied for Shc expression using DNA microarray data and immunohistochemistry. ShcKD (hypomorph) and age-matched wild type (WT) mice were pair-fed according to the chronic-plus-binge alcohol (NIAAA) diet. To analyze hepatocyte-specific effects, AAV8-TBG-Cre (ShcHepKO)-mediated deletion was performed in fl/fl Shc mice. Lipid peroxidation, proinflammatory signals, redox radicals, NADH/NAD+ ratio, as well as cleaved caspase 8, BAP31, Bax, and Bak, in vivo. CRT translocation was studied in ethanol-exposed p46ShcδSH2-transfected hepatocytes by membrane biotinylation in conjunction with p-Eif2α, BAP31, caspase 8, Bax/Bak. The effects of idebenone, a novel Shc inhibitor was studied in alcohol/pair-fed mice.

Results: Shc was significantly induced in patients with AH (p<0.01). ALT, NADH/NAD+ ratios, production of redox radicals, lipid peroxidation improved (p<0.05), and IL-1β, MCP-1, and CXCL10 were reduced in ShcKD and ShcHepKO mice. In vivo, Shc-dependent induction, and in hepatocytes, p46Shc-dependent increase in pre-apoptotic proteins Bax/Bak, caspase 8, BAP31 cleavage and membrane translocation of CRT/Erp57 were seen. Idebenone protected against alcohol-mediated liver injury.
**Conclusion:** Alcohol induces p46Shc-dependent activation of pre-apoptotic pathways and translocation of CRT to the membrane, where it acts as a DAMP, instigating immunogenicity. Shc inhibition could be a novel treatment strategy in AH.

**Keywords:** alcoholic hepatitis, Shc, calreticulin, lipid peroxidation, sterile inflammation
Introduction

Alcoholic hepatitis (AH) is a severe form of alcoholic liver disease (ALD) that carries high mortality. Clinically, it is characterized by severe inflammation, rapid decline in liver synthetic function, and often death. The pathogenesis of AH has distinct features with lipid peroxidation, severe redox stress, sterile inflammation, monocytic and neutrophil recruitment. As to how these processes are interlinked, however, has not been well understood.

The group of Src homology and collagen (Shc) proteins are important adapters to extra-cellular signals, and are involved in aging-related pathways[1], regulate receptor tyrosine kinase (RTK)[2], and insulin signaling[3]. Shc proteins derive from the ShcA locus: p46 and p52Shc are generated from different start codons, whereas p66Shc is a product of alternative splicing. The p66Shc isoform has been studied in alcohol-induced liver injury in vitro and in animal experiments[4, 5]. However, the mouse models used were not always clear p66Shc KOs, and the other isoforms were reduced, as well (hypomorphs). In addition, the other mitochondrial isoform, p46Shc exhibits more substantial induction upon alcohol treatment, therefore further studies are needed to delineate its role in the context of alcoholic hepatitis. Calreticulin (CRT), an endoplasmic reticulum (ER)-resident protein that is involved in Ca^{2+} signaling, upon danger signals exits the ER, and translocate to the membrane to act as a damage associated molecular pattern (DAMP) molecule signaling for recognition by immune cells[6, 7]. CRT as a DAMP has not been studied in AH, and given the redox sensitive adapter role of Shc, we postulated that Shc-mediated oxidative signals and lipid peroxidation may lead to the activation of pre-apoptotic pathways in response to alcohol, prompting CRT translocation[8, 9].

In this study, our goal was to decipher how Shc-mediated CTR membrane translocation is involved in generating pre-apoptotic and proinflammatory signals in alcoholic hepatitis, and whether Shc inhibition could represent a novel therapeutic approach.
Results

Shc is induced in patients with alcoholic hepatitis (AH)

To study Shc in aged-matched AH patients, first we analyzed DNA microarrays[10]. SHC1 gene expression was induced in the livers of AH patients compared to livers from healthy subjects (Fig. 1A). Immunohistochemistry (IHC) on liver biopsy samples from patients with AH revealed increased Shc signal mainly in hepatocytes, in contrast to healthy controls (Fig. 1B). Consistently, the liver Shc mRNA transcripts was positively associated with serum ALT levels in our mouse model of alcoholic liver injury, which will be further discussed below (Fig. 1C).

Shc knockdown (hypomorph, ShcKD) and hepatocyte-specific Shc knockout (ShcHepKO) mice are protected against alcohol-induced liver injury and oxidative stress

To study the role of Shc in AH, we first evaluated ShcKD mice that exhibit a decrease in all Shc isoforms (hypomorphs; total Shc deletion is embryonically lethal). Mice were pair-fed chronic-plus-binge alcohol diet (NIAAA protocol)[11]. p66Shc was very low at baseline and did not show induction following the NIAAA diet (Fig. 2A). ShcKD mice had significantly lower serum ALT levels compared to WT mice on NIAAA diet (Fig. 2B). Alcohol is known to be oxidized by alcohol dehydrogenase and acetaldehyde dehydrogenase and increase the reduced nicotinamide adenine dinucleotide (NADH)/ oxidized nicotinamide adenine dinucleotide (NAD+) ratio in the cytosol and mitochondria of hepatocytes[12], and this was seen in our study. In alcohol-fed ShcKD mice however, the NADH/NAD+ ratio significantly decreased (Fig. 2C). Lipid peroxidation as studied by 4-hydroxynonenal (4-HNE) showed lower signal in ShcKD mice (Fig. 2D).

As we localized Shc predominantly to hepatocytes and confirmed that liver NADH/NAD+ ratios were affected by Shc knockdown, next we studied the role of hepatocyte Shc in alcoholic...
liver injury. Shc^{fl/fl} mice were injected with either adeno-associated virus 8 (AAV8)-Cre on day 5 of the 10d diet to delete Shc in hepatocytes, or by AAV8-control virus as control (Fig. 3A). Shc^{HepKO} (mice injected with AAV-8-Cre) mice had improved histology, lower lipid peroxidation (4-HNE signal, Fig. 3B), and significantly lower serum ALT levels (Fig. 3C) compared to mice injected with the control virus. In addition, NIAAA diet induced production of reactive oxygen species (ROS) (Fig. 3D) and NADH/NAD\(^+\) ratios (Fig. 3E), and these were significantly lower in Shc^{HepKO} mice. Liver triglyceride (TG) assay and oil red o staining indicated that liver steatosis mildly improved in Shc^{HepKO} mice (Figure. 3F&G).

**Shc^{KD} and Shc^{HepKO} mice exhibit lower levels of inflammatory mediators**

Immunohistochemistry (IHC) depicted lower number of F4/80 positive monocytic cells in Shc^{HepKO} mice on NIAAA diet(Fig. 4A). RT-qPCR showed that monocyte chemoattractant protein 1 (MCP-1), C-X-C chemokine ligand 10 (CXCL10), and interleukin 1 \( \beta \) (IL-1\( \beta \)) were blunted in Shc^{KD} (Fig. 4B), and in Shc^{HepKO} mice (Fig. 4C). Luminex multiplex assay revealed MCP-1, IL-4, CXCL-10, and macrophage colony-stimulating factor (M-CSF) reduction in Shc^{HepKO} mice (Fig. 4D).

**Alcohol induces pre-apoptotic changes and calreticulin translocation in a Shc-dependent pathway**

Plasma membrane exposure of the ER-resident chaperon CRT as a DAMP has been linked to immunogenic cell death[13]. Sterile inflammation is an important early pathogenic feature of AH therefore it was tempting to speculate that CRT could be involved in mediating pre-apoptotic signals contributing to immunogenicity.

Alcohol is well-recognized to activate different branches of the ER stress response[14][15]. Calreticulin translocation as an early event can be mediated by ER stress signals leading to an induction of pre-apoptotic pathways with caspase 8-dependent cleavage of B-cell receptor-associated protein 31 (BAP31), and activation of Bax and Bak[16, 17]. Indeed, cleaved
caspase-8 (p41/p43, p18), BAP31 (p20), and Bax and Bak were seen in control virus-injected mice on NIAAA diet while these were attenuated in ShcHepKO mice (Fig. 5A). To better capture CRT translocation, primary hepatocytes from WT and ShcKD mice were treated with ethanol. The signal in alcohol-treated hepatocytes exhibited membrane pattern however this was much lower in ShcKD hepatocytes (Fig. 5B). To further study CRT translocation in human cells we used the VL-17A cells, (HepG2 cells that express cytochrome p450 2E1 and alcohol dehydrogenase)[18]. Ethanol treatment resulted in CRT translocation to the cell surface (Fig. 5C). ERp57 is a stress-responsive protein in the disulfide isomerase family that mainly resides in ER. CRT/ERp57 co-translocation to the cell membrane signifies immunogenicity of cell death[19], therefore we studied their surface presence in VL-17A cells after ethanol exposure. We isolated cell surface proteins by biotinylation, and found that both CRT and ERp57 increased after 16h ethanol treatment (“Biotinylated”), while the total protein level (“Input”) remained similar over time (Fig. 5D).

The P46Shc isoform is involved in modulating pre-apoptotic signals after alcohol exposure

Shc proteins are known to modulate mitochondrial redox signaling, and the p66Shc isoform has been studied; however, in the liver its expression was low at basal or alcohol-fed conditions. Therefore, we focused on the role of the mitochondrial p46Shc isoform that was induced by the NIAAA diet. The Src homology 2 binding domain (SH2) has been described essential to p46Shc binding and dysregulating 3-ketoacyl-CoA thiolase (ACAA2) activity, and thereby inhibiting mitochondrial β-oxidation of fatty acids[20]. Indeed, markedly reduced ACAA2 activity was observed in alcohol-exposed rat livers[21]. Therefore, we transfected primary hepatocytes with p46ShcΔSH2 (Fig. 6A) or control plasmid, and exposed cells to alcohol. In p46ShcΔSH2-transfected, alcohol-exposed cells most of the CRT signal remained perinuclear, compared to mock-transfected alcohol-exposed cells where we observed cell membrane
exposure, as before (Fig. 6B). Calreticulin translocation can be mediated by ER stress response involving phosphorylation of the eukaryotic translation initiation factor 2 subunit alpha (EIF2S1) and also specific pre-apoptotic events with caspase-8-activation[16, 17]. In the mock-transfected alcohol-exposed cells EIF2s1 was induced and activated; and we observed cleavage of caspase 8, and BAP31, as well as Bax and Bak induction. These effects of ethanol treatment were attenuated in p46ShcδSH2 transfected cells (Fig. 6C). In addition, alcohol-induced calreticulin translocation was blunted in VL-17A cells transfected with siBAX (Fig. 6D, E).

**Shc inhibition protects against alcohol-induced liver injury in vivo**

As p46Shc-specific inhibitors are not available, next we evaluated the efficacy of pharmaceutical Shc inhibition in mice on the NIAAA diet. Idebenone, a benzoquinone was discovered to have a potent Shc inhibitory effect by directly interacting with Shc to block its phosphorylation[22]. Mice on NIAAA diet were dosed with idebenone (20 mg/kg, once daily). This group exhibited significantly reduced serum ALT (Fig. 7A). The expression of inflammatory mediators Cxcl1 and F4/80 (Adgrel) were attenuated by idebenone (Fig. 7B), and liver histology improved, as well (Fig. 7C). Additionally, in mouse primary hepatocytes idebenone pretreatment (10 µM, 30 min) abrogated alcohol-induced calreticulin translocation (Fig. 7D).

**Discussion**

In this study we show that during alcoholic hepatitis, Shc activation, particularly the p46Shc isoform, lipid peroxidation and CRT membrane exposure are interlinked (Fig. 8). The Shc family of adapter proteins are involved in modulating redox and metabolic stress signals elicited by various stimuli e.g. receptor tyrosine kinases (RTK), or growth factor signaling. Of the three isoforms, the liver mainly express p46Shc, p52Shc in physiological conditions whereas the level of p66Shc is much lower[23]. In the past most Shc-related effects were
attributed to p66Shc, however the knockout mice used in these experiments in reality were hypomorphs having reduced levels of all three isoforms; therefore, the other isoforms deserve attention[3]. In our study, we observed that Shc was induced in patients with alcoholic hepatitis, and that feeding of NIAAA diet to mice led to increased expression of p46Shc and p52Shc. We focused on p46Shc as it is a mitochondrial isoform and was shown to bind to and inhibit the enzymatic activity of the lipid oxidation enzyme ACAA2[20]. Thus, it was plausible that p46Shc induction during AH could result in reduced β-oxidation, that in turn causes increase in ROS and lipid peroxidation. Indeed, we found that oxidative radicals and lipid peroxidation improved in Shc$^{HepKO}$ and Shc$^{KD}$ mice fed the NIAAA diet. These effects of course, could have been the cumulative result of reducing all isoforms as studying p46Shc-specific effects in vivo is not yet possible. We have earlier shown that in non-alcoholic steatohepatitis, p52Shc could bind to and activate the NADPH oxidase 2, via its p47phox subunit, inducing production of ROS[23]. Therefore, it is conceivable that in alcoholic hepatitis not only the mitochondrial isoforms but p52Shc also plays a role in generation of redox radicals.

Alcohol metabolism exerts a significant increase in NADH/NAD$^+$ ratio in the mitochondria and cytoplasm of hepatocytes. This is thought to be linked to a defect in β-oxidation, and in our case the ratio was reversed in Shc$^{KD}$ and Shc$^{HepKO}$ mice, signifying an important Shc-mediated effect. Taken together, these data point to an important maladaptive effect elicited by increased Shc in alcoholic liver disease, and furthermore detailed studies are required to decipher the effects of p46Shc on mitochondrial bioenergetics, and lipid metabolism.

Alcohol-induced lipid peroxidation can result in proteostasis with an activation of endoplasmic reticulum (ER) stress[14]. ER stress involving EiF2α activation and caspase 8-mediated BAP31 cleavage were linked to the activation of Bak and Bax in cancer cells, and depleting caspase-8, BAP31, Bax, Bak prevented CRT/ERp57 exposure and immunogenicity[24]. As both EiF2α activation and pre-apoptotic signals were attenuated after p46ShcδSH2
transfection, p46Shc is likely to be an important isoform in alcoholic injury. CRT membrane presence can signal immunogenicity even before the cells manifest real signs of apoptosis, and this could be an important feature in early injury in alcoholic hepatitis, as the presence of CRT on the cytoplasmic membrane is a key signal for recognition by mononuclear cells[25]. We found that chemokines denoting monocytic recruitment e.g. MCP-1, IL-4, CXCL-10, were significantly reduced in a Shc-dependent manner, in both the Shc\textsuperscript{KD}, and Shc\textsuperscript{HepKO} livers and after treatment with the Shc inhibitor. Macrophages are known to play a proinflammatory role and tissue remodeling in alcohol models[26]. In our study, consistently, number of the F4/80, cells increased and Shc deletion in hepatocyte and idebenone treatment reversed the effects. Idebenone was recently discovered as a potent Shc inhibitor, albeit not isoform-specific[22]. For a long time idebenone was thought to be an antioxidant however recent data reveal potent binding activity at nanomolar concentrations to Shc[27]. Idebenone in our study reduced ALT/AST, inflammatory mediators and CRT expression.

Taken together, in alcoholic hepatitis Shc-dependent pre-apoptotic pathways in conjunction with ER stress lead to membrane exposure of CTR/Erp57 as an important DAMP complex. Targeting Shc could be a novel therapeutic strategy mitigating liver injury in alcoholic hepatitis.

Materials and Methods

Human Biopsy Samples

DNA microarray data were extracted and analyzed from NCBI GSE28619[10]. The DNA microarray included 15 AH patients (mean age 49-year-old) and 7 normal livers (mean age 51-year-old). Formalin-fixed paraffin-embedded human liver biopsy samples were obtained from University of California (UC) Davis Cancer Center Biorepository (funded by the National Cancer Institute). Samples were de-identified and exempted (Exemption 4). The study protocol
conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee. Biopsy samples from 3 different patients were analyzed by immunohistochemistry (IHC).

**Animal Studies**

All animal experiments were conducted according to the experimental procedures approved by the Institutional Animal Care and Use Committee at UC Davis, Stanford University, and Palo Alto, VA. Shc hypomorph (“Shc knockdown, ShcKD”) mice were originally from Dr. Pelicci’s laboratory[1]. Shc<sup>0/0</sup> mice were gifted by Dr. Fawaz Haj (UC Davis, CA). Genotypes of the progenies were confirmed as previously described[1, 28]. Mice were housed in standard cages with 12:12 hour light/dark cycles and *ad libitum* access to water and food unless otherwise indicated. Chronic plus binge alcohol treatment was given as per the NIAAA protocol[29]. In brief, all mice were first acclimated to 5-day Lieber-DeCarli control liquid diet (Bio-Serv, #F1259SP). Mice in the alcohol (NIAAA) group were subjected to 10-day 5% v/v ethanol (Fisher, #BP2818-500)-containing liquid diet (Bio-Serv, #F1258SP) and an acute ethanol binge (5g/kg body weight) on the 11<sup>th</sup> day, and mice in control (Pair-fed) group were fed with control liquid diet and binged with maltose dextrin in a calorie-matched manner. In the first cohort, 14-week-old Shc<sup>KD</sup> mice and age-matched Shc<sup>0/0</sup> (WT) mice were placed on NIAAA model or subjected to hepatocyte isolation. In the second cohort, 14-week-old male Shc<sup>0/0</sup> mice were assigned to NIAAA group or Pair-fed group in an age-matched version, and were i.v. injected with either adeno-associated virus 8 (AAV8)-control green fluorescent protein (AAV8-control) or AAV8-thyroxine-binding globulin-Cre recombinase (AAV8-Cre) on day 5 of the 10-day diet to generate hepatocyte-specific Shc-knockout mice (5x10<sup>11</sup> genome copies, Vector BioLabs), and the rationale of the specific deletion in hepatocyte has been previous reported[30]. In the third cohort, 10-week-old C57BL6 mice (Jackson Laboratory) were assigned to NIAAA group or Pair-fed group and dosed with idebenone (MilliporeSigma,
as previously described[22]. In brief, mice were acclimated before the 10-day NIAAA diet started, then were fed either 0.11 gr of peanut butter (vehicle group, idebenone’s absorption is increased by food high in lipids) or idebenone formulated in 0.11 gr of peanut butter (20 mg/kg body weight, idebenone group) once daily for 10 days. Mice were observed eating all the peanut butter.

Cell Culture

Primary mouse hepatocytes were isolated following collagenase reverse perfusion as previously described[31]. The cells were maintained in William’s E medium (Sigma-Aldrich, #W4125) supplemented with 10% fetal bovine serum (FBS) (Gibco, #10437028) and antibiotics (Gibco, #15240062). VL-17A cells were gifted by Dr. Wen-Xing Ding (University of Kansas Medical Center) and Dr. Dahn Clemens (University of Nebraska Medical Center), selected by G418 (Gibco, #10131035) and Zeocin (Invitrogen, #R25001) and cultured in DMEM medium (Gibco, #11995073) supplemented with 10% FBS and antibiotics.

Cell Transfection, Plasmids, and siRNAs

Plasmids containing human wild-type p46Shc and deletion mutant p46ShcδSH2, were constructed and confirmed (Dr. Tomilov[20]). SH2 domain was deleted using forward primer

5’-GAGCTGCTCAGCCATGGACAC-3’ and reverse primer 5’-GAGCGGAATTCTACCTCGAGT-3’, and EX-H9090-M62-p46Shcδ (329–425) SH2 was constructed. Primary hepatocytes were transfected with 2 µg DNA per 12-well or 3 µg DNA per 6-well for about 24 hours (h) using jetPEI-Hepatocyte DNA transfection reagent (Polyplus, #89129-944) following the manufacturer’s instruction. BAX siRNA (#sc-29212) and control siRNA (#sc-37007) were from Santa Cruz Biotechnology, INC., and the transfection was performed using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, #L3000001) according to manufacturer’s instructions. Briefly VL-17A cells were seeded on a collagen-
coated 8-well chambered glass slide and incubated with 10 pm BAX or control siRNA and 1 µl Lipofectamine 3000 reagent in serum-free medium for 24 h.

**Serum ALT Measurement**
Mouse blood was collected at the time of euthanasia and centrifuged at 8000 g at room temperature (RT) for 10 minutes (min) for 2 times. The supernatant was collected as serum, aliquoted, and snap-frozen. ALT and AST levels were measured by UC Davis Comparative Pathology Lab or Diagnostic Laboratory at Stanford Department of Comparative Medicine.

**Liver TG Measurement**
Frozen liver tissue (20 to 50 mg) was homogenized in 1 mL of chloroform/methanol (v/v = 2:1) with vigorous shaking for 1 hour at room temperature. Subsequently 200 µL of Millipore water was added into the mix and centrifuged at 3000 × g for 5 min. The lower lipid phase was collected and dried in a fume hood overnight, and the pellet was dissolved in 60 µL of tert-butanol and 40 µL of Triton X-114/methanol (v/v = 2:1) mix. Liver TG levels were measured with colorimetric assay kits, following the manufacturer’s instructions (Pointe Scientific, Canton, MI), and normalized to tissue weight. The tert-butanol and Triton X-114/methanol mixture was used as a blank control.

**RNA Extraction, Reverse Transcription, and Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)**
Total RNA was isolated from snap-frozen liver tissue and cells as per the manufacturer’s instructions using RNeasy mini kit (Qiagen, #74104). Equal concentration of RNA was used to synthesize complementary cDNA using iScript™ cDNA synthesis kit (Bio-Rad, #1708891). RT-qPCR was done using the Power SYBR Green PCR Master Mix (Applied Biosystems, #4368706) on 7900HT system (Applied Biosystems), and the results were
analyzed by $2^{-\Delta \Delta Ct}$ method. The results were normalized with acidic ribosomal phosphoprotein ($Arbp$) as endogenous control. The primer sequences used in this study are listed in Table 1.

**Protein Extraction and Western Blotting**

Frozen liver tissue samples were homogenized and lysed with radioimmunoprecipitation (RIPA) buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140mM NaCl). The homogenate was centrifuged and the supernatant was collected for western blot. Cells were washed with phosphate buffered saline (PBS) and lysed with Cell Lysis Buffer (Cell Signaling Technology, #9803). The homogenate was centrifuged and the supernatant was collected. Protein concentration was determined with Bio-Rad Protein Assay Kit (Bio-Rad, #5000001) or Pierce BCA Protein Assay Kit (Thermo, #23225). Protease inhibitor (Roche, #4693116001) and phosphatase inhibitor (Roche, #4906837001) were added to all the lysis procedures mentioned above, and 10-50 µg of the protein samples were loaded onto SDS-polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane or nitrocellulose membrane which was blocked with 5% BSA in TBST and then incubated with primary antibodies at 4°C (Table 2) overnight. The blots were washed with TBST and further incubated with horseradish peroxidase-conjugated secondary antibodies (Table 3). Signal was detected by adding Western-Bright enhanced chemiluminescence substrate (Advanta, Menlo Park, CA, Cat#K-12045-D20) or SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo, #34596) and imaged with film or iBright CL1500 imaging system (Thermo). The images were processed and analyzed with NIH ImageJ and iBright Analysis software (Thermo).

**Histology, Immunohistochemistry, Immunofluorescence, and Oil Red O staining**

Paraffin-embedded tissue samples were cut into 5 µm sections, deparaffinized, and rehydrated. For antigen retrieval, slides were boiled in citrate buffer (0.01 M, pH 6.0) using microwave oven on high power for 5 min and cooled down to room temperature. After incubation in 3%
aqueous H₂O₂ to quench the endogenous peroxidase, sections were washed in PBST (PBS with 0.1% Tween 20, v/v) washing buffer, blocked with 5% goat serum (EMD Millipore, #566380) diluted in PBST at RT for 1 h, and incubated with primary antibody (Table 2) diluted in 2% goat serum in PBST at 4°C overnight. All slides were scanned with Leica Aperio AT2 at Stanford Human Pathology /Histology Service Center. Images were processed with NIH ImageJ.

For immunohistochemistry, slides were incubated with appropriate biotinylated secondary antibodies (Table 3) for 1 h, then with the ABC Peroxidase Standard Staining Kit (Thermo, #32050) for 30 min. The slides were stained with 3, 3’ Diaminobenzidine (Abcam, #ab64238) for 5 seconds (sec) to 5 min and counterstained with hematoxylin (Thermo, #72704) for 45 sec.

For immunofluorescence on tissues, slides were incubated with Alexa fluor secondary antibodies (Table 3) at RT for 1 h. Slides were washed with PBST between incubation and mounted with antifade mounting medium with DAPI (VectorLabs, #H-1200). Fluorescent images were taken with Leica TCS SPE (Leica Microsystems Inc.) at the Stanford Cell Sciences Imaging Facility (NIH SIG 1S10OD010558001A1).

Oil red O staining was performed on cryosections from fresh liver tissues embedded and frozen in Optimal Cutting Temperature Compound (Scigen). The slides were fixed in 10% neutral buffered formalin (Research Products International) for 30 min. After washing with water, the slides were placed in 100% propylene glycol (Poly Scientific) for 5 min and 85% propylene glycol for another 5 min. The slides were stained for oil red o stain (Frontier Scientific) preheated at 60°C for 10 min, and differentiated in 85% propylene glycol for 3 min. The slides were counterstained with hematoxylin stain, Harris for 30 sec and bluing solution for 10 dips.
Fluorescent Immunocytochemistry

Primary hepatocytes were plated on collagen-coated cover slips. After treatment, cells were washed twice with PBS and fixed with 4% paraformaldehyde at 4°C overnight. Cells were permeabilized in PBS with 0.4% (v/v) Triton X-100 for 10 min. After blocking with 5% goat serum in PBST at RT for 1 h, cells were incubated with primary antibodies (Table 2) diluted in 2% goat serum in PBST at 4°C overnight. The following morning the slides were washed and then incubated with secondary antibodies (Table 3) at RT for 1 h.

For surface staining, cells were washed with PBS twice, fixed with 0.25% paraformaldehyde for 5 min, and incubated with primary antibodies and secondary antibodies for 30 min respectively. Coverslips were washed with PBST between incubations and mounted with anti-fade mounting medium with DAPI. Fluorescent images were taken with Leica TCS SPE. Images were processed with NIH ImageJ.

NADH/NAD Assays

Snap-frozen liver tissue samples were processed with the NADH/NAD assay kit (Abcam, #ab65348) as per manufacturer’s protocol. Briefly, total NAD (including NAD⁺ and NADH) were extracted from liver tissues with extracting buffer. Equal amount of protein was subjected to the assay.

Lucigenin Assay

Lucigenin assay was performed as described previously[32]. Snap-frozen liver were homogenized on ice in sucrose buffer (0.3 M sucrose, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM Spermidine, 1 mM DTT, pH 7.4) with protease inhibitor, and centrifuged at 1000 g for 5 min. The supernatant was further centrifuged at 100,000 g for 1 hour at 4°C to obtain membrane-enriched fractions. The pellet was suspended in Krebs buffer (100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.0 mM K₂HPO₄, 25 mM NaHCO₃, 20 mM Na-HEPES, 0.2% glucose, pH 7.4) with protease inhibitor and incubated at 37°C for
Membrane fractions were incubated with 10 µM lucigenin (Invitrogen, #L6868) at room temperature for 15 min. Then 100 µM of NADPH (Sigma-Aldrich) was added and the chemiluminescence intensity was read with Pharmingen Monolight 3010 luminometer (BD) every 1 minute, up to 10 counts. Data were normalized to the protein concentration.

**Luminex 39-plex Cytokine Assay**

Frozen mouse livers were homogenized and same protein concentrations were solubilized in RIPA buffer supplemented with protease inhibitor. Luminex cytokine measurement was performed by Immunoassay Team at the Human Immune Monitoring Center at Stanford University. Mouse 39 plex Procarta kit (Thermo) was used according to the manufacturer’s recommendations with slight modifications. Net median fluorescent intensity was used to generate heatmaps and to perform statistical analysis.

**Surface Protein Isolation, and Biotinylation**

For surface protein isolation, cells were plated and processed using the Pierce Cell Surface Protein Isolation Kit (Thermo, #89881) following the manufacturer’s instructions. In brief, cells were labelled with EZ-Link Sulfo-NHS-SS-Biotin, lysed, protease inhibitor and phosphatase inhibitor added (“input”), isolated with NeutrAvidin Agarose column, and eluted with SDS sample buffer (Bio-Rad, #161747) containing 50 mM DTT “eluate”. Samples were subjected to western blotting as described above. No-Stain Protein Labeling Reagent (Invitrogen, #A44449) was added as control.

**Statistical Analysis**

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA, USA) using unpaired t test with Welch’s correction or Mann-Whitney test for non-parametric values. Normality distribution was assessed with Kolmogorov-Smirnov test. A value of at least p<0.05 has been considered and stated as significant.
All authors had access to the study data and had reviewed and approved the final manuscript.

Acknowledgements

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References


Figure Legends

**Figure 1. Shc is induced in patients with alcoholic hepatitis (AH).** DNA array studies from 7 healthy subjects (NL, normal liver) and 15 patients with severe alcoholic hepatitis (AH) showed significant induction of Shc in AH (A, mean±SEM, **p<0.01, data base: GSE28619, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28619). Immunohistochemistry for Shc was performed on biopsy samples from 3 healthy normal livers and 3 AH patients (B, bar=100 µm). The correlation coefficient of serum ALT levels and liver Shc mRNA levels (normalized to Arbp) was calculated (C, simple linear regression analysis, $R^2=0.5033, p<0.001$).

**Figure 2. Shc knock-down (KD) in vivo improves lipid peroxidation, redox injury, ALT, and NADH/NAD$^+$ ratios.** Wild-type (WT) and Shc hypomorph (Shc$^{KD}$) mice were pair-fed NIAAA diet. Western blot analyses (A) depicted that mainly the p52Shc and p46Shc isoforms were induced by the NIAAA diet, and in Shc$^{KD}$ mice these were attenuated (A, Densitometry data presented as mean ± SEM, **p<0.01). Serum ALT levels (B) and liver NADH/NAD$^+$ ratios (C) were significantly increased in WT mice on the NIAAA diet, but not in Shc$^{KD}$ mice. (B&C: n=5-9 mice per group.) 4-hydroxynonenal (HNE) immunohistochemistry showed decreased signal in Shc$^{KD}$ mice compared to WT mice following alcohol feeding (D, bar=100 µm)(All data are presented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.)
Figure 3. AAV8-cre mediated deletion of Shc from hepatocytes ($Shc^{HepKO}$) in vivo improves lipid peroxidation, redox injury, lowers ALT levels, and NADH/NAD$^+$ ratios.

To study hepatocyte Shc, $Shc^{fl/fl}$ mice were placed on the NIAAA/pair-fed diet. On day 5 of the 10d diet they were injected with either AAV8-Cre, or AAV8 control ($5 \times 10^{11}$ genome copies, each). Western blot shows Shc expression in the different models, densitometry depicts total Shc (A). Hematoxylin eosin (H&E) and 4-hydroxynonenal (4-HNE) depict improved histology and lipid peroxidation, respectively (B, bar=100 µm). Serum ALT values (C) and redox injury (lucigenin assay) (D) show improvement, and while the NADH/NAD$^+$ ratios were induced in NIAAA-treated-, control virus-transduced mice, these significantly improved in Shc hepatocyte-deleted mice (E). Liver TG values (F) and Oil Red O staining (G) show mild improvement in Shc$^{HepKO}$ mice. (C-E: n=6-9 mice per group.) (All data are presented as mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.)

Figure 4. Shc knock-down (KD) or deletion of Shc from hepatocytes ($Shc^{HepKO}$) improves inflammation. Immunohistochemistry for F4/80 depicts lower number of positive cells in livers of AAV8-cre injected $Shc^{fl/fl}$ mice ($Shc^{HepKO}$) on NIAAA diet compared to AAV8-control-injected mice (A, bar=100 µm, five 20x fields/mouse in 3 mice were counted). RT-qPCR demonstrated NIAAA diet-induced MCP-1, CXCL10, IL-1β, and TNF-α in WT mice, but this was attenuated in Shc$^{KD}$ mice (B, n=5-8/group). NIAAA diet induced MCP-1, IL-1β, TNFα, and CXCL10 in control virus-transduced mice, these were attenuated in Shc$^{HepKO}$ mice, as assessed by RT-qPCR (C, n=6/group). Liver homogenates were analysed by Luminex 39-plex assay and the representative heatmap is shown (D). Compared to pair-fed mice, most mediators were induced on NIAAA diet, and lower levels of MCP-1, IL-4, CXCL10, and M-CSF were seen in Shc$^{HepKO}$ mice. (All data are presented as mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.)
Figure 5. Alcohol induces the translocation of calreticulin (CRT) in a Shc-dependent manner. Western blots on liver cytosolic proteins depict that cleavage of caspase-8 and BAP31 in Shc^0/0^ mice on NIAAA diet, but not in Shc^HepKO^ mice; and Bcl2 members Bax and Bak were attenuated in Shc^HepKO^ mice (A). Immunofluorescence studies of primary hepatocytes isolated from control (WT) or Shc^KD^ livers show CRT localized to the perinuclear area (indicated by white arrows) in non-treated cells (B, red=CRT, blue=DAPI, bar=20 µm). After ethanol treatment (EtOH, 100 mM for 8 hours), CRT signal was observed on the cell membrane in WT (indicated by blue arrowheads), but not in the Shc^KD^ hepatocytes. Quantitation of the number of membrane-positive cells revealed significant increase in alcohol-treated WT cells (five 20x fields per group were counted, mean±SEM, *p<0.05, **p<0.01). To study CRT translocation in human cells, VL-17A cells were treated with 100 mM ethanol for 8 hours, and immunofluorescence done, as above. Increase in cell membrane exposure of CRT (indicated by white arrows) was seen in alcohol-treated cells (C, red=CRT, blue=DAPI, bar=10 µm). The surface membrane proteins of VL-17A cells were labelled with biotin after 100 mM ethanol treatment for 8 or 16 hrs. Whole cell lysate (“Input”) and isolated cell surface proteins (“Biotinylated”) were subjected to western blotting (D). There was an increase in cell surface CRT and ERp57 at 16h of alcohol exposure.
Figure 6. p46Shc inhibition decreases calreticulin cell membrane translocation and alcohol-mediated induction of EIF2S1, caspase 8 cleavage and activation of Bak and Bax. Mouse primary hepatocytes were transfected with the p46ShcδSH2 plasmid- or mock-transfected for 24 hrs (A), exposed to alcohol (100 mM for 8 hours), and immunofluorescence was performed to visualize calreticulin. In mock transfected, alcohol-exposed cells calreticulin exhibited an increased cell membrane signal (white arrows), but not in p46ShcδSH2-transfected cells (B, red=calreticulin, blue=DAPI, bar=20 µm). In p46ShcδSH2-transfected cells subjected to western blot analysis, phos-EIF2S1 (Ser51), cleaved caspase 8, BAP31, Bak and Bax were attenuated (C). VL-17A cells were transfected with control siRNA (siCont) or BAX siRNA (siBAX) for 24 hrs, exposed to alcohol (100 mM for 24 hrs), and immunofluorescence was performed to visualize calreticulin. In alcohol-exposed cells calreticulin exhibited an increased cell membrane signal (white arrows) in siCont- but not in siBAX-transfected cells (D, red=calreticulin, blue=DAPI, bar=20 µm). % of surface CRT+ cells were quantified (E, data are presented as mean ± SEM, n=4-7 10x fields per group, **p<0.05).
Figure 7. Idebenone, a Shc inhibitor protects against alcohol-induced liver injury in vivo. Mice on alcohol diet (NIAAA model) were treated with idebenone 20 mg/kg or vehicle once daily for 10 days. Serum ALT levels were significantly reduced by idebenone (A, n=7-8/group). RT-qPCR to evaluate pro-inflammatory transcripts demonstrated decreased expression of CXCL1 and F4/80 (encoded by Adgre1) in idebenone-treated mice (B, n=6-8/group). Hematoxylin eosin (H&E) showed improved inflammation, and steatosis (C, bar=100 µm). Primary mouse hepatocytes were treated with idebenone 10 µM for 30 min followed by alcohol (EtOH) 100 mM, and immunofluorescence was performed to visualize calreticulin. In alcohol-exposed cells calreticulin exhibited an increased cell membrane signal (white arrows), but not in idebenone-pretreated cells (D, red=calreticulin, blue=DAPI, bar=50 µm). (All data are presented as mean ± SEM, n=6-8, *p<0.05.)

Figure 8. Schematic depiction of the proposed role of Shc and calreticulin (CRT) translocation in alcohol-induced injury in hepatocytes. Shc induction during alcoholic hepatitis is linked to maladaptive responses with increased lipid peroxidation and production of reactive oxidative species (ROS). Key to this is the mitochondrial p46Shc isoform that can inhibit fatty acid β-oxidation. Activation of EIF2S1, pre-apoptotic signals with caspase-8 and BAP31 cleavage, and Bak/Bax activation lead to CRT/ERp57 complex translocation from the endoplasmic reticulum to the cell membrane where they act as DAMP and elicit an inflammatory response. Shc inhibition may protect hepatocytes against alcohol-induced injury. (Created with BioRender.com)
### Table 1. Sequence of primers used in this study.

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<th>Primer Sequence</th>
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Table 2. List of primary antibodies used for various applications in the study.

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<td>WB: 1:1000, IF: 1:250, FACS: 1:500</td>
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Table 3. List of secondary antibodies used in the study.

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<td>Biotinylated Goat α-Rabbit</td>
<td>Vector Lab, #BA-1000</td>
<td>IHC, 1:500</td>
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