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Combination of Alcohol and Cigarette Smoke Induces Endoplasmic Reticulum Stress and Cell Death in Pancreatic Acinar Cells

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Abstract

Background & Aims—Smoking, an independent risk factor for pancreatitis, accelerates the development of alcoholic pancreatitis. Alcohol feeding of mice induces upregulation of spliced X-box binding protein 1 (XBP1s), which regulates the endoplasmic reticulum (ER) unfolded protein response and promotes cell survival upon ER stress. We examined whether smoking affects the adaptive mechanisms induced by alcohol and accelerates disorders of the ER in pancreatic acinar cells.

Methods—We studied the combined effects of ethanol and cigarette smoke extract (CSE) on ER-stress and cell death responses in mouse and human primary acini and the acinar cell line AR42J. Cells were incubated with ethanol (EtOH, 50 mM), CSE (20–40 μg/ml), or both (CSE+EtOH), and analyzed by immunoblotting, quantitative reverse transcription PCR, and cell death assays. Some cells were incubated with MKC-3946, an inhibitor of endoplasmic reticulum to nucleus signaling 1 (ERN1, also called IRE1) that blocks XBP1s formation. Male Sprague-Dawley rats were fed isocaloric amounts of an ethanol-containing (Lieber-DeCarli) or control diet for 11 weeks and exposed to cigarette smoke or room air in an exposure chamber for 2 hours each day. During the last 3 weeks, a subset of rats received intravenous injections of lipopolysaccharide (LPS, 3 mg/kg

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per weeks) to induce pancreatitis or saline (control). Pancreatic tissues were collected and analyzed by histology and immunostaining techniques.

Results—In AR42J and primary acini, CSE+EtOH induced cell death (necrosis and apoptosis), but neither agent alone had this effect. Cell death was associated with a significant decrease in expression of XBP1s. CSE+EtOH, but neither agent alone, slightly decreased ATP levels in AR42J cells, but induced oxidative stress and sustained activation (phosphorylation) of eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3, also called PERK) and increased protein levels of DNA damage inducible transcript 3 (DDIT3, also called CHOP). CHOP regulates transcription to promote apoptosis. Incubation of AR42J or primary mouse or human acinar cells with MKC-3946 reduced expression of XBP1s, increased levels of CHOP and induced cell death. In rats fed ethanol diet, exposure to cigarette smoke increased ER stress in acinar cells and sensitized the pancreas to LPS-induced pathology.

Conclusions—Cigarette smoke promotes cell death and features of pancreatitis in ethanol-sensitized acinar cells by suppressing the adaptive unfolded protein response signaling pathway. It also activates ER stress pathways that promote acinar cell death.

Keywords

alcohol; smoking; ER stress; pancreas

Introduction

Alcohol abuse and smoking are key risk factors for pancreatitis and pancreatic cancer.[1-3] The increased risk for pancreatic cancer in alcohol abusers occurs largely through the effect of alcohol acting as a causal factor for acute and chronic pancreatitis. Smoking independently contributes to pancreatitis (estimated up to 3-fold excess risk),[2] and, compared to non-smokers increased by 2-fold the risk of pancreatic cancer independent of pancreatitis.[3-5] Epidemiologic studies indicate that smoking may promote pancreatitis in alcoholic patients with these two environmental stressors having an additive effect on the initiation and progression of the disease.[1-5] The mechanisms mediating the potential interactions between alcohol abuse and smoking that result in pancreatic injury are incompletely understood.

We showed that ethanol feeding regulates signals of the endoplasmic reticulum (ER) Unfolded Protein Response (UPR).[6] The UPR is a set of concerted signaling pathways that monitors ER events including proper protein folding, and regulates the expression of numerous genes that support ER homeostasis.[7] The main types of UPR responses are: 1) enhancement of the ER capacity to handle unfolded proteins, 2) reduction of the protein load entering the ER, 3) activation of protein degradation programs to rid the ER of accumulated unfolded/misfolded proteins; and 4) activation of cell death signaling under conditions of severe and/or prolonged ER stress.[6, 8]

Distinct UPR branches sense disturbances in ER homeostasis and operate in a coordinated manner through unique signaling pathways. The Inositol requiring enzyme 1 (IRE1) branch is the most conserved. Activation of IRE1 endoribonuclease leads to splicing of the mRNA

encoding XBP1u (unspliced X-box binding protein 1), generating the transcription factor XBP1s (spliced form). XBP1s target genes include many ER/Golgi chaperones and foldases, vesicular trafficking factors and enzymes regulating lipid synthesis.[9] XBP1s also promotes the development of an extensive ER network characteristic of secretory cells including acinar cells.[10] Early induction of XBP1s upon ER stress is critical for ER adaptation to stress and cell survival, while premature termination of this signal leads to ER dysfunction and cell death.[11, 12]

Activation of the protein kinase RNA-like ER kinase (PERK) branch of the UPR upon ER stress leads to phosphorylation of translation initiation factor (eIF2α) and broad inhibition of mRNA translation, which when well-tuned and transient, is thought to contribute to a protective UPR. However, persistent PERK activation induces upregulation of C/EBP homologous protein (CHOP),[7] a transcription factor that controls genes involved in apoptosis and inflammation. Thus, with more significant and prolonged activation, the PERK branch of the UPR contributes to programmed cell death.

Our previous study[6] demonstrated that ethanol feeding causes ER oxidative stress and XBP1s upregulation in pancreas without a change in PERK or CHOP; and that preventing the increase in XBP1s leads to PERK activation, CHOP upregulation, ER pathology and acinar cell death. Thus, alcohol activates an adaptive UPR involving XBP1s and this adaptive response may account for the fact that only a small number of individuals develop overt pancreatitis with alcohol abuse.[13]

This study sought to determine whether smoking can perturb or curtail the adaptive mechanisms induced by alcohol and accelerate disorders of the ER. We examined UPR signals in acinar cells after stimulation with a combination of ethanol and cigarette smoke extract (CSE) and observed that CSE reduced XBP1s protein levels. This effect was associated with features of severe ER stress, such as prolonged PERK activation, increased CHOP expression, and reduced cell survival. In an animal model of alcoholic pancreatitis elicited by co-administration of alcohol and LPS, we found that addition of smoking caused exacerbation of the pancreatitis response, as evidenced by marked ER morphologic changes on electron microscopy associated with increased CHOP levels. We concluded that alcohol and smoking promote the development of alcoholic pancreatitis by inducing dysregulation of the UPR and proteostasis mechanisms in the acinar cell.

Methods

Reagents

Ethanol (#459836) was purchased from Sigma-Aldrich (Saint Louis, MO) and cigarette smoke extract (#CSE) from Murthy Pharmaceuticals (Lexington, KY). CSE is prepared by burning University of Kentucky 3R4F Standard Research Cigarettes on a Filter Smoke Machine. The cigarette smoke is condensed, stored in a filter, extracted with DMSO by soaking and sonication, and then packaged as 40 mg total extract/ml (equivalent to 3.6 cigarettes per ml). Detailed information about CSE and other reagents is provided in Supplementary Methods.

Cell Culture

The rat acinar cell line AR42J (ATCC® CRL1492™; ATCC, Rockville, MD) was used for short- and long-term culture studies (up to 96 h). Cells were cultured and differentiated with dexamethasone as indicated in Supplementary Methods. treated with ethanol (EtOH; 50 mM), cigarette smoke extract (CSE; 20-40 μg/ml) or the combination of both. In addition, cell cultures were treated with acrolein (25-40 μM), a chemical found in cigarettes in high concentrations, alone or in combination with ethanol. Further, AR42J cells were treated with the Aryl hydrocarbon receptor (AhR) ligand 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; 0.5 nM) and the AhR antagonist CH-223191 (0.5-20 μM) to determine whether the AhR pathway participates in CSE- and EtOH-induced effects.

Freshly isolated mouse and human pancreatic acini were used for short-term studies (up to 48 h). Mouse acini were isolated from untreated male C57CL/6 mice. Human acini were isolated from cadaveric pancreatic tissue from organ donors (both sexes) at City of Hope (Duarte, CA). Mouse and human acini were treated for 24-48 h with ethanol, CSE or both, and with other experimental agents as indicated above for AR42J cells and detailed in Supplementary Methods.

Animal model of alcoholic pancreatitis with smoke exposure

Male Sprague-Dawley rats were fed isocaloric amounts of control (C) or ethanol-containing Lieber-DeCarli diet (A) for a total of 11 weeks. During this period, control- or ethanol-fed animals were exposed to either cigarette smoke or room air in an exposure chamber for 2 hours each day (smoke concentration within the chamber was adjusted to simulate heavy smoking i.e. 25-30 cigarettes/day). During the last three weeks, a subset of rats received LPS (3 mg/kg per week; intravenously) to induce pancreatitis or saline as control. Eight groups of rats were studied: (C) control-fed exposed to room air; (CS) control-fed exposed to smoke; (A) alcohol-fed exposed to room air; (AS) alcohol-fed exposed to smoke (AS); (CL) control-fed exposed to room air and injected with LPS; (AL) alcohol–fed exposed to room air and injected with LPS; (CLS). control-fed exposed to smoke and injected with LPS; and (ALS) alcohol–fed exposed to smoke and injected with LPS. Rats were euthanized 24 h after the last LPS injection, and pancreas tissues processed for measurements of pancreas pathology and ER stress. Additional details are provided in Supplemental Methods.

Morphometry for pancreatic tissue lesions

Paraformaldehyde-fixed, paraffin-embedded rat pancreatic tissues were H&E or Sirius red stained for assessment of pancreas pathology and fibrosis. Cell apoptosis and neutrophil infiltration into the pancreas were analyzed by TUNEL and IHC for myeloperoxidase, respectively.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used as an indicator of cellular metabolic activity and viability in AR42J cells treated with ethanol and/or CSE. Details are provided in Supplementary Methods.

Cell Death Characterization

Cell death induced by ethanol and cigarette smoke extract in AR42J cells and primary mouse and human acini was measured by propidium iodide uptake. Apoptosis and necrosis were further characterized by Annexin-V-FLUOS staining (#11858777001, Roche Molecular Biochemicals) in living cells. Apoptosis was also assessed by measuring internucleosomal DNA fragmentation in cell lysates using Cell Death Detection ELISAPLUS kit (#11774425001, Sigma-Aldrich). Detailed information is provided in Supplementary Methods.

GADD153/CHOP staining

GADD153/CHOP protein expression and nuclear location, as indicative of ER stress-related apoptosis, was evaluated in paraformaldehyde-fixed AR42J cells by conventional immunofluorescence techniques as described in Supplementary Methods.

Electron microscopy evaluation of cultured cells and rat pancreatic tissues

Acinar cell ultrastructure was examined by electron microscopy (EM). Cultured cells and pancreatic tissues from rats subjected to alcoholic pancreatitis and smoking were fixed with 2.5% glutaraldehyde and examined by EM as described in Supplementary Methods.

Amylase secretion

Dexamethasone-treated AR42J cells were stimulated for 1 h with cholecystokininoctapeptide (CCK-8, 10 nM), and then amylase secretion into the conditioned media was measured using the Phadebas test (Pharmacia Diagnostic, NY) as previously described.[6] Data were expressed as the percentage of amylase activity detected in the conditioned medium relative to the total amylase activity (medium+cells).

ATP luminescence assay

Cells were treated for up to 48 h as indicated in the Results section. Total cellular ATP levels were measured in cell extracts using a bioluminescence ATP determination Kit (#A22066, ThermoFisher Scientific) per the manufacturer's recommendations.

Biological oxidation measurement

Protein oxidation in ethanol- and CSE-treated AR42J cells was assessed by labeling cell protein extracts with BIAM (biotin-conjugated iodoacetamide (N-(biotinoyl)-N-(iodoacetyl)-ethylene-diamine) as described in Supplementary Methods.

Western blot analysis

Protein extracts from cultured cells and rat pancreatic tissues were analyzed using standard Western blotting techniques; specifics and antibody information are found in Supplementary Methods.

RNA analysis by qPCR

Total RNA from AR42J cells was extracted using the RNeasy® Plus Mini Kit (#74034; Qiagen, CA). Reverse transcription and quantitative real-time PCR (qPCR) was performed as indicated in Supplementary Methods.

Statistical analyses

All experiments were performed in triplicate unless otherwise stated. Data are presented as mean±SEM. Data were subjected to analysis of variance (ANOVA) followed by Tukey's post-hoc test, and two tailed *Student t* test for comparison between two groups. *P* values <0.05 were considered significant.

Results

Ethanol and smoking compounds in combination decrease cell viability and induce cell death in acinar cells

Clinical and experimental evidence indicate that heavy drinking and smoking are associated with pancreas pathology. To determine whether exposure to both ethanol and cigarette smoke extract (CSE) induces toxicity in pancreatic acinar cells, we treated AR42J cells for up to 96 h with CSE (10-40 μg/ml) and ethanol (EtOH; 50 mM) alone and in combination. The concentrations of the experimental agents were chosen to reproduce in culture conditions the levels of exposure to alcohol and smoking compounds that can be achieved in acinar cells after prolonged heavy drinking and smoking. Phase-contrast microscopy revealed similar morphology in AR42J cells treated with EtOH or CSE alone to that of untreated cells. In contrast, cells treated with CSE+EtOH displayed morphologic changes consistent with cell death, including rounding and detachment from dishes, cell shrinking and condensation of cytoplasm (Supplementary Figure 1). Since the toxic effects of the combined treatment were observed with CSE at 20 μg/ml and more effectively at 40 μg/ml, we selected 40 μg/ml as the CSE concentration for the remainder of the study.

To characterize the toxic effects of the combined treatment, we measured metabolic activity in AR42J cells by MTT assay. (Figure 1A). Compared to control cells, treatment with EtOH for 96 h slightly increased MTT values, while CSE treatment reduced metabolic activity by 15%. Most importantly, there was a marked decrease in cell viability with CSE+EtOH that was significantly greater than the CSE alone. Measurements of propidium iodide (PI) uptake, an indicator of loss of plasma membrane integrity and cell death, revealed a significant increase in cell death in AR42J treated with CSE+EtOH compared to the individual treatments (Figure 1B). Similarly, 24-h treatment with CSE+EtOH increased PI uptake in mouse pancreatic acinar cells, whereas the individual treatments had no effect (Supplementary Figure 2).

To characterize the cell death induced by EtOH and CSE, we measured apoptosis by DNA fragmentation ELISA assay, and both apoptosis and necrosis by immunostaining with Annexin-V/PI. Based on these measurements, EtOH or CSE alone had minor effects on acinar apoptosis and necrosis (Figure 1C and D). However, as measured by Annexin-V staining and DNA fragmentation assay, CSE+EtOH markedly increased apoptosis. There

was also a significant increase in secondary necrosis (Annexin-V/PI positive cells, Figure 2C), but apoptosis was the main cell death mechanism induced by CSE+EtOH.

Ethanol and CSE in combination induce marked activation of the PERK pathway in acinar cells

To determine whether ethanol and smoking effects on cell death responses were associated with ER stress and pathological UPR, we measured protein levels of p-PERK and p-eIF2α in acinar cells (Figure 2). We monitored the PERK UPR branch because we previously found that in XBP1 deficient mice fed ethanol diet, the pancreas exhibited acinar cell pathology associated with sustained activation of PERK and upregulation of CHOP.[6] AR42J cells were treated with the experimental agents for up to 72 h, whereas primary acinar cell treatments were limited to 24 h since these cells rapidly dedifferentiate in culture. In AR42J cells, EtOH induced a transient mild activation of PERK that peaked at 48 h, while CSE induced a higher and more sustained activation that persisted at 72 h (Figure 2A). Compared to the individual treatments, CSE+EtOH caused higher and persistent activation of PERK leading to sustained phosphorylation of eIF2α, an event linked to global blockade of protein synthesis. Similar activation of PERK was found in primary cultures of mouse acini (Figure 2C).

CSE increases levels of pro-apoptotic regulators in acinar cells

To ascertain whether triggering of the PERK pathway has an impact on cell death signaling, we measured protein levels of CHOP and cleaved caspase 3 in ETOH- and CSE-treated AR42J cells. Addition of CSE+EtOH to AR42J cells resulted in marked upregulation of CHOP and significant increases in levels of active, cleaved caspase 3 (Figure 3A and B), while the individual treatments had no effect. To determine cellular localization, we measured immunofluorescence staining for CHOP in 72 h-treated AR42J cells. As illustrated in Figure 3C, there was no evidence of CHOP immunoreactivity in control and EtOH-treated cells, and few CSE-treated cells displayed weak nuclear positive staining. In contrast, CSE+EtOH treated cells showed significant positive staining in condensed nuclei, suggesting CHOP transcriptional activity. These data suggest that ethanol and smoking compounds induce acinar cell death in an ER-stress dependent manner.

PERK activation induced by ethanol and CSE is associated with oxidative stress but not with a decrease in cellular ATP

Protein folding within the ER is highly sensitive to changes in the ER redox status,[14] and both reducing and pro-oxidant agents induce accumulation of misfolded proteins and UPR activation.[15] ER protein folding is governed by electron transfer between protein disulfide isomerases, the ER oxidoreductase ERO1 and molecular oxygen, a process that generates reactive oxygen species (ROS).[14] Previous studies showed that CHOP induces ERO1 upregulation leading to accumulation of ROS and exacerbation of ER stress.[16] Besides a controlled redox state, ER function also requires energy in the form of ATP. ATP modulates many ER processes, including protein folding, chaperoning function, protein degradation and ER-to-Golgi protein export, and, accordingly, ATP depletion triggers UPR activation.[17]

To determine whether ATP depletion mediates the observed effects of CSE+EtOH on ER stress, we measured total cellular ATP levels in AR42J cell lysates. We found that, irrespective of the treatment, ATP levels remained constant within 24 h after treatments, with minor decreases (10-15%) in cells treated with CSE or CSE+EtOH for 48 h (Figure 4A), a time point when we found robust ER stress and CHOP upregulation (Figure 3). We did not monitor ATP levels within the ER, but the data suggest that ATP depletion per se is not the major cause of ER stress induced by ethanol and CSE.

To show a potential role for oxidative stress we used the BIAM assay, which detects free sulfhydryl moieties on proteins, such that oxidation of the sulfhydryl groups diminishes BIAM incorporation. The extent of BIAM incorporation into proteins was monitored by streptavidin blot analysis. AR42J cell treatment with CSE+EtOH led to noticeably increased oxidation of cysteine residues, as shown by reduced biotin-labeling. To further demonstrate that CSE+EtOH promotes oxidative stress, we measured expression of the oxidative-stress response genes NAD(P)H:Quinone Oxidoreductase 1 (Nqo1), Heme oxygenase 1 (Hmox1) and Glutamate-Cysteine Ligase (Gclc). CSE+EtOH significantly upregulated Nqo1 and Hmox1, and to a lesser extent, Gclc (Figure 4C). These results imply that oxidative stress at least partially mediates the ER stress and PERK activation we see in our study. Consistent with this, the antioxidant N-acetylcysteine (NAC) reduced PERK activation and cell death induced by CSE+EtOH in acinar cells (Supplementary Figure 3). Taken together, the data support our hypothesis that smoking and alcohol cause pancreatic damage by dysregulation of the cellular redox state and by prolonging and exacerbating ER stress.

CSE reduces XBP1s expression in pancreatic acinar cells

Widespread evidence supports the concept that upon ER stress the IRE1/XBP1s branch of the UPR mediates adaptive responses to reestablish cellular homeostasis. XBP1s deficiency or premature attenuation of IRE1/XBP1s signaling during ER stress promotes sustained activation of PERK/CHOP and cell death.[6,12,18] Moreover, sustained IRE1/XBP1 activity enhance cell survival in conditions of severe stress,[11] further supporting a protective role for this pathway. We reported that pancreatic acinar cells express XBP1s in physiological conditions, and that *Xbp1* genetic deletion in mice impairs digestive enzyme secretory responses in acinar cells, and decreases the number of zymogen granules in pancreas of ethanol-fed mice.[6] These data demonstrate a key role for XBP1s in maintaining ER function and the secretory pathway in the acinar cell.

We found here that AR42J expressed XBP1s in basal conditions, and EtOH increased these levels by 15% (Figure 5A and Supplementary Figure 4). Interestingly, CSE reduced XBP1s levels by 40% compared to controls, and by 60% compared to EtOH treated cells. CSE effects on XBP1 expression were associated with a decrease in zymogen granule formation (Figure 5B-D and Supplementary Figure 5), as would be expected due to the key role of XBP1s to maintain the differentiated state of the acinar cell.

We next asked whether CSE-induced XBP1s deficiency participates in the cell death responses we observed in CSE+EtOH-treated cells. To address this, we used a specific inhibitor of the IRE1-RNase (MKC-3946; here IRE1-I) that blocks XBP1s formation.[19] AR42J cells were preincubated with either IRE1-I or vehicle, then exposed to EtOH and

CSE alone or in combination. IRE1-I reproduced the effects of CSE+EtOH treatment on XBP1s levels and CHOP upregulation in AR42J cells (Figure 6A). In addition, AR42J exposure to IRE1-I recapitulated in all groups (control, EtOH-, CSE- or CSE+EtOH-treated) the same degree of cell death observed in cells treated with CSE+EtOH alone (Figure 6B). Similar results were obtained in primary mouse and human acinar cells (Figure 6C and D). These results strongly imply that CSE-mediated reduction in XBP1s levels leads to CHOP upregulation and cell death in cells treated with CSE+EtOH. Moreover, whereas stimulation of AR42J cells with cholecystokinin (CCK) increased XBP1s expression, IRE1-I inhibition of XBP1s formation attenuated CCK-induced secretion of the digestive enzyme amylase and reduced protein levels of a marker of the ER network, calnexin (Supplementary Figure 4). These results indicate that appropriate XBP1s levels are required for normal secretory responses in acinar cells, and may explain the loss of ER network in cells treated with CSE +EtOH (Figure 5C).

The precise molecular mechanisms underlying CSE-induced XBP1s downregulation remain unknown. That treatment with IRE1-I to block XBP1s formation mimics CSE effects raises the possibility that chemicals in CSE inhibit IRE1 in a similar manner as IRE1-I. IRE1-I contains an aldehyde motif that inhibits IRE1-RNase by forming a reversible Schiff base with Lys907 in the catalytic site.[19] CSE contains many aromatic compounds and reactive chemicals, including unsaturated aldehydes such as acrolein, (estimated 60-300 μg per cigarette). [20] Acrolein has been found to form adducts with DNA and proteins in the lungs of chronic smokers,[21] and also reacts with Lys541 and Lys545 in albumin.[22] Acrolein was shown to induce protein adduct formation, and to mimic ethanol-induced ATF4/CHOP activation and cell death in cultured hepatocytes.[23] We found here that acrolein alone or in combination with ethanol dose-dependently induces acinar cell death, associated with XBP1s downregulation and CHOP upregulation (Supplementary Figure 6). Independently, a previous study showed that the CSE component hydroquinone causes apoptosis in retinal cells by upregulating CHOP and supressing XBP1s.[24] Taken together, accumulating data indicate that CSE chemicals such as acrolein and hydroquinone may promote cell death via UPR dysregulation.

Effects of smoking in an animal model of alcoholic pancreatitis

The findings presented above suggest that smoking reduces adaptive UPR signals, leading to alcohol-mediated injury of acinar cells. To test the hypothesis that these effects of smoking would promote pancreatitis, we employed a well-established rat model of alcoholic pancreatitis.[25] In this model, pancreatitis manifests when alcohol feeding is accompanied by repeated administration of lipopolysaccharide (LPS), a factor elevated in the circulation of heavy drinkers due to increased gut permeability.[26] We used a custom-built smoke chamber housing a rack of individual cages that allows simultaneous administration of cigarette smoke in the atmosphere and ethanol feeding. Smoke exposure was calibrated such that exposed animals exhibited serum nicotine levels of 653.6±60.1 ng/ml, corresponding to levels expected in heavy smokers.[27]

Histologic data (Figure 7 and Supplementary Figure 7) indicate that, while LPS administration to control-fed rats (CL) had no effect (not shown), an alcohol diet (A)

significantly induced pancreatic damage when co-administered with LPS (L), as we previously reported.[25] Notably, smoking (S) further increased the severity of pancreatitis in alcohol-fed, LPS challenged rats (ALS group). Thus, we observed increases in histologic scores for inflammatory cell infiltration, fibrosis (confirmed by Sirius red staining), acinar cell vacuolization and cell death (necrosis + apoptosis), in pancreas from ALS, as compared to the AS and AL groups. Neutrophil infiltration was significantly elevated by LPS in both the AL and ALS groups (Supplementary Figure 7D). Interestingly, acinar cell apoptosis was increased in the AS group, in the absence of LPS-induced inflammation, and LPS further augmented this response. (Supplementary Figure 7C).

We previously reported that alcohol diet induces ultrastructural changes in the acinar cell indicative of ER stress, mainly ER dilation and vesiculation, and that these features are exacerbated in XBP1-deficient mice.[6,13] To further establish the effects of smoking in the pathologic ER stress response, we examined pancreas tissues by electron microscopy. We found that, irrespective of other treatments, ER dilation was a common feature in pancreas from alcohol-fed rats (Supplementary Figure 8). However, animals in the ALS group displayed severe ER dilation and vesiculation, and reduced numbers of mature zymogen granules, as expected in conditions of impaired UPR and ER dysfunction. Consistent with these data, alcohol+LPS induced classic UPR activation that involves both increases in protective XBP1s signal and PERK/CHOP activation (Figure 7E and F). However, inclusion of smoking treatment (ALS group), as we found in cultured acinar cells, produced an impaired UPR response characterized by CHOP upregulation but limited increases in XBP1s protein levels. These data support our hypothesis that smoking promotes pancreas damage by promoting a dysfunctional UPR response to ER stress induced by heavy drinking.

CSE+EtOH-induced effects on acinar cell death and XBP1 expression are not mediated by aryl hydrocarbon receptor (AhR) signaling

Numerous aromatic compounds including those in cigarette smoke are AhR ligands, and experimental evidence indicates that the AhR participates in the development of smoking-related disorders including cancer.[28] A recent report demonstrated that AhR ligands promote fibrosis in a mouse model of chronic pancreatitis.[29]

To determine whether AhR activation mediates CSE effects in our experimental models, acinar cells were treated with an antagonist of AhR (CH-223191) or the prototypical AhR ligand TCDD alone or in combination with CSE and EtOH. Results are shown in Supplementary Figure 9. After 48 h treatment, CSE induced marked activation of AhR, as shown by upregulation of the AhR target genes Cyp1a1 and Cyp1a2; TCDD or TCDD +EtOH further increased expression of both genes. Similar results were found after 72 h incubation (not shown). However, in contrast to CSE or CSE+EtOH, TCDD or TCDD +EtOH treatments did not reduce XBP1s expression. Further, pretreatment with the AhR antagonist abrogated CSE- or TCDD-induced upregulation of Cyp1a1a and Cyp1a2, but left intact the effects of CSE+EtOH on XBP1s expression and cell death.

In our rat model of alcoholic pancreatitis, exposure to cigarette smoke increased pancreatic Cyp1a1 mRNA levels in ethanol-fed rats (group AS; Supplementary Figure 10), indicating AhR activation. However, in rats treated with LPS (AL and ALS groups), an agent that

promoted pancreatic inflammation and fibrosis, Cyp1a1 levels were similar to control-fed rats. The mechanisms whereby LPS suppresses AS-induced increases in Cyp1a1 expression are unclear. Taken together, our data suggest that smoking-induced AhR activation elicits signaling pathways that operate independently from those involved in UPR dysregulation and cell death.

Discussion

Unresolved ER stress underlies the pathology of many chronic diseases including ethanol-related disorders.[30] In the liver, ER stress responses were linked to ethanol-induced protein misfolding, hyperhomocysteinemia leading to homocysteinylation of ER proteins, and activation of proapoptotic signals downstream to the PERK/CHOP branch.[31] Ethanol metabolism also causes lipid peroxidation, oxidative stress and ER dysfunction in liver, brain and other organs.[32] We have reported that chronic ethanol feeding to rodents mobilizes adaptive ER stress responses in exocrine pancreas involving the IRE1/XBP1 UPR branch. [6,33] In the absence of this adaptive response there is impaired early onset of protective IRE1/XBP1s signaling, and upregulation of the PERK/CHOP pathway that converge into ER dysfunction and acinar cell pathology that mimics several features of human alcoholic pancreatitis. Our data provide insight into the complex mechanisms whereby chronic alcohol exposure and other environmental factors alter ER function and induce proteostasis disorders in the acinar cell.

The contribution of ER stress to the development of smoking-related disorders is increasingly being appreciated. Cigarette smoke contains a complex mixture of over 7,000 potentially harmful components, including oxidants, aldehydes, and aromatic hydrocarbons; these molecules can damage proteins, lipids, and nucleic acids. In this context, functional adaptive responses are critical to protect cells from toxic smoking compounds. Accumulating evidence indicates that smoke extracts induce ER stress and cell death in lung epithelial cells, inflammatory cells and other cell types.[34-37] In lung epithelial cells, smoking was linked to oxidative stress, reduced proteosomal-dependent protein degradation, ER stress and CHOP induction, and apoptosis.[38] In lung cancer cells, smoking compounds induced UPR activation that led to upregulation of several genes with key roles in attenuating ER oxidative stress and providing survival advantages to these cells.[36, 39] Salient data from our study suggest that in acinar cells smoking compounds exacerbate ethanol-induced oxidative ER stress causing protein misfolding and UPR activation. Cycles of protein misfolding and refolding in the ER may further increase the generation of reactive oxygen species (ROS) and exacerbate ER dysfunction. ROS can likely impose multiple effects on ER resident and folding proteins, which will be important to determine to understand the pathogenesis of alcohol abuse and smoking-induced pancreatitis.

We show here that unlike ethanol, CSE causes inhibition of XBP1s formation in acinar cells, promoting activation of the PERK/CHOP pathologic ER stress pathway and cell death. Indeed, pharmacological inhibition of XBP1s formation mimicked the CHOP-dependent acinar cell death induced by EtOH+CSE. Inhibition of XBP1s also attenuated stimulated enzyme secretion in AR42J cells, supporting a key role for XBP1s in maintaining the secretory phenotype and homeostatic programs in the acinar cell. Our data are consistent

with previous reports indicating that CSE dysregulates the UPR and suppresses XBP1s in several cell types, and that overexpression of XBP1 protects cells against CSE-induced apoptosis.[24, 35] The mechanisms underlying the inhibitory effects of smoking on XBP1s expression in acinar cells are unclear and may be related to decreases in gene transcription, XBP1 RNA stability, or inhibition of IRE1-induced XBP1 RNA splicing. In this respect, we found that acrolein, a CSE chemical that, like the IRE1-RNase inhibitor, can form Schiff bases with lysine residues and protein and DNA adducts, [22] also reduces XBP1s levels and promotes cell death. Thus, acrolein may be among a range of reactive chemicals in CSE that contribute to inhibition of IRE1-RNase activity and/or DNA damage.

Our study indicates that smoking induced a robust activation of AhR signaling in acinar cells, but this was dispensable for CSE+EtOH-induced XBP1s downregulation and acinar death induction. The AhR is a ubiquitous nuclear factor that mediates toxic and/or adaptive responses in a cell-type and ligand-specific manner.[40] In a chronic pancreatitis model, AhR activation in T cells elicited IL-22 secretion associated with promotion of the fibrotic pancreatic stellate cell phenotype.[29] Given the robust activation of AhR by smoking compounds in acinar as well as immune cells, the specific role of AhR on adaptive/inflammatory responses in different cell types participating in smoking-related pancreatic disorders deserves further investigation.

Importantly, we show that smoking promotes pancreatitis responses *in vivo*. In an established model of alcoholic pancreatitis, smoking caused dramatic ER dilation and vesiculation. These hallmarks of ER stress were accompanied by a dysregulated UPR response similar to that found in cultured acinar cells, i.e. attenuated XBP1s concomitant with upregulated CHOP and acinar cell apoptosis. Whether smoking also promotes inflammatory signals in our animal model is unclear. While the total number of inflammatory cells within H&E-stained pancreas tissues was highest in the ALS group, the number of MPO positive cells was similarly elevated in the AL and ALS groups. Further studies are needed to clarify the effects of smoking on inflammatory signals in alcoholic pancreatitis models.

Taken together, our study identifies central regulators of the ER stress response as key participants in the pathogenesis of chronic pancreatitis associated with heavy drinking and smoking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A	alcohol diet
AL	alcohol diet + LPS

ALS	alcohol diet + LPS + smoking
AS	alcohol diet + smoking
Acro	acrolein
AhR	Aryl hydrocarbon receptor
C	control diet
CHOP	C/EBP homologous protein
CSE	cigarette smoke extract
EtOH	ethanol
ER	endoplasmic reticulum
eIF2α	eukaryotic translation initiation factor 2α
LPS	lipopolysaccharide
PERK	protein kinase RNA (PKR)-like ER kinase
XBP1s	spliced X-box-binding protein-1
XBP1u	unspliced X-box-binding protein-1
UPR	unfolded protein response

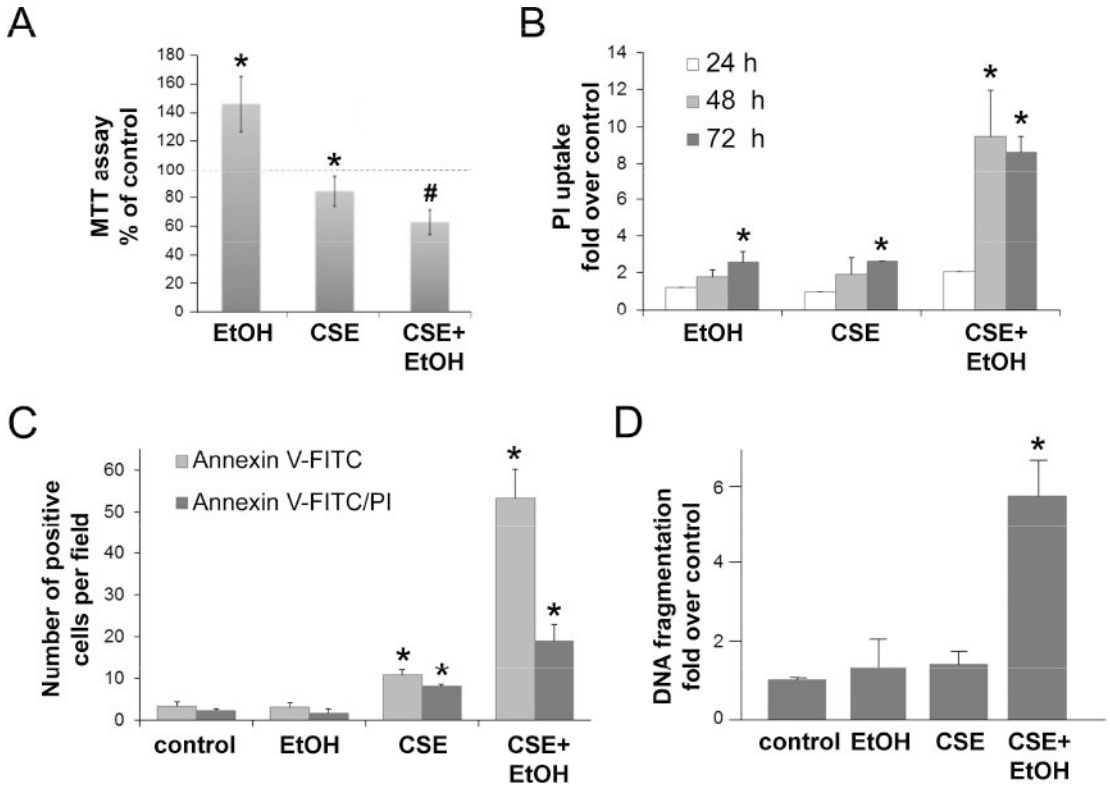

Figure 1. Ethanol and CSE in combination decreased cell viability and induced cell death

AR42J cells were treated for up to 96 h with ethanol (EtOH, 50 mM) or CSE (40 μg/ml) alone or in combination. (A) Percentage of viable cells (measured by MTT assay) at 96 h relative to control. Data shows mean±SEM, n=3-4; **P*<.05 vs. control; #*P*<.05 vs. CSE or EtOH. (B) As determined by propidium iodide (PI) uptake, CSE+EtOH induced a significant increase in cell death compared to control, EtOH or CSE treatments. The data are mean ±SEM, n=3; **P*<.05 vs. control. (C) Apoptosis and necrosis after 72 h treatment were assessed by staining cells with Annexin V-FITC and PI, respectively. Positive cells were counted under a confocal microscope; at least 8 random fields were analyzed per group. Cell death was judged according to staining with Annexin V-FITC alone (early apoptosis), both Annexin V-FITC and PI (late apoptosis) or to PI only (necrosis). Data are expressed as mean ±SEM; n=3. * *P*<.05 vs. control. (D) Apoptosis was confirmed by measuring internucleosomal DNA fragmentation by ELISA (Roche). Data in graph are mean±SEM; 3 independent experiments. **P*<.05 vs. control.

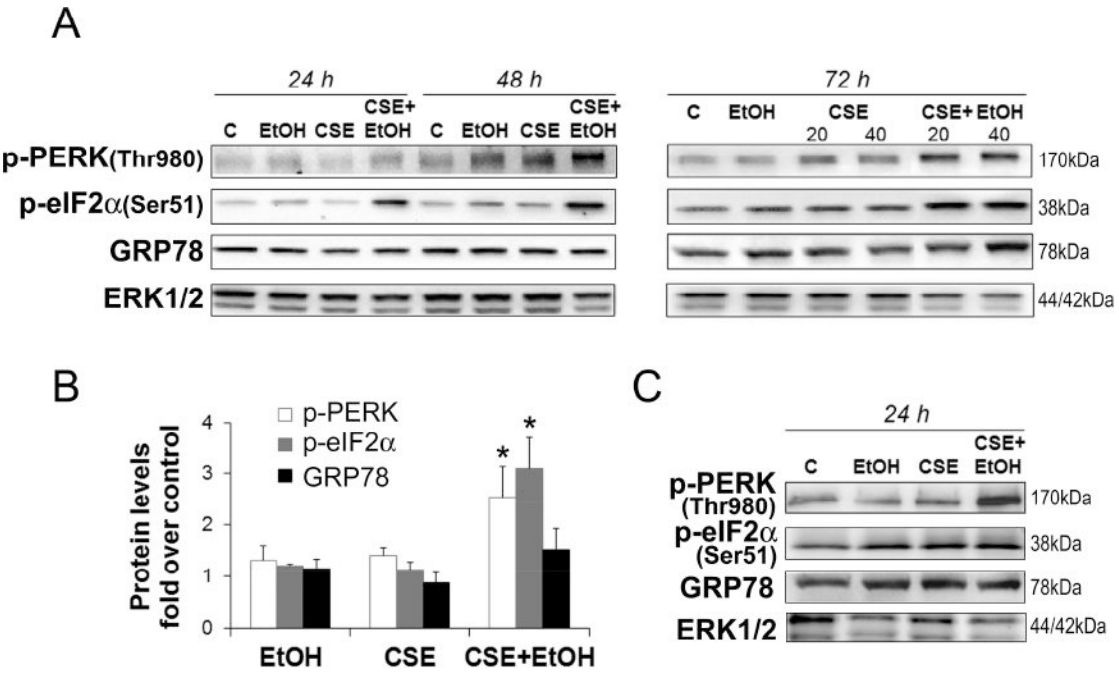

Figure 2. Ethanol and CSE in combination induce marked activation of the PERK pathway in acinar cells

(A) AR42J cells were left untreated (C) or treated for the indicated times with EtOH, (50 mM) and/or CSE (40 μg/ml, left panel; 20 or 40 μg/ml, right panel). Expression levels of the UPR markers phospho-PERK, phospho-eIF2α, and GRP78 were assessed by Western blotting. Blots are representative of 3 independent experiments. (B) Densitometry analysis of immunoblots from (A), 48 h treatment. Levels (as a fraction of control values) were normalized to those of ERK1/2. Graph shows mean±SEM, n=3; * P<.05 vs. control. (C) Mouse pancreatic acini were treated for 24 h with 50 mM EtOH and/or 40 μg/ml CSE. Immunoblots show protein levels of phospho-PERK, phospho-eIF2α, GRP78, and ERK1/2.

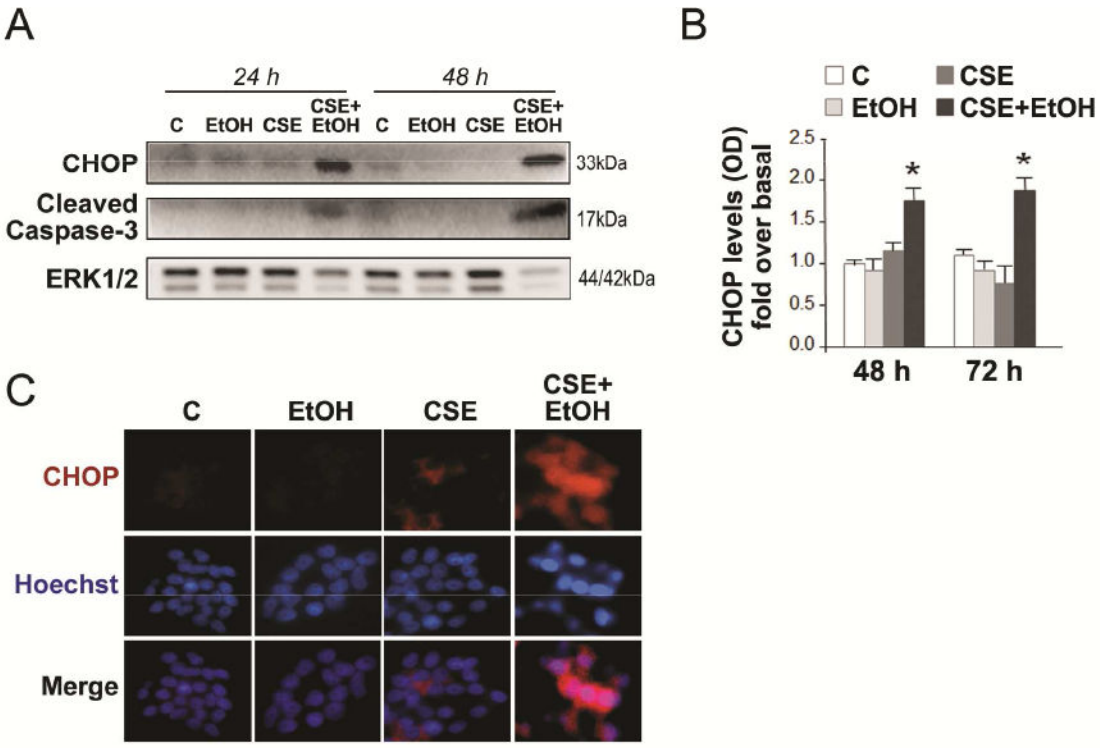

Figure 3. Combination of ethanol and CSE treatment induces upregulation of CHOP and its translocation to the nucleus

AR42J cells were left untreated (C) or treated with 50 mM ethanol (EtOH) and/or 40 μg/ml CSE for the indicated times. (A) Immunoblots show protein levels of the apoptotic regulators CHOP and caspase-3. (B) Graph shows quantitation of CHOP relative to ERK1/2 bands in immunoblots. Data is mean±SEM, n=3; * P<.05 vs. control. (C) Immunofluorescence staining of CHOP (red staining) in cells treated for 72 h with EtOH and CSE alone or in combination. Nuclei were counterstained with Hoechst 33342; n=2 independent experiments. As shown in the panels, CHOP upregulation and nuclear location were evident only in cells treated with CSE and, especially, with CSE+EtOH.

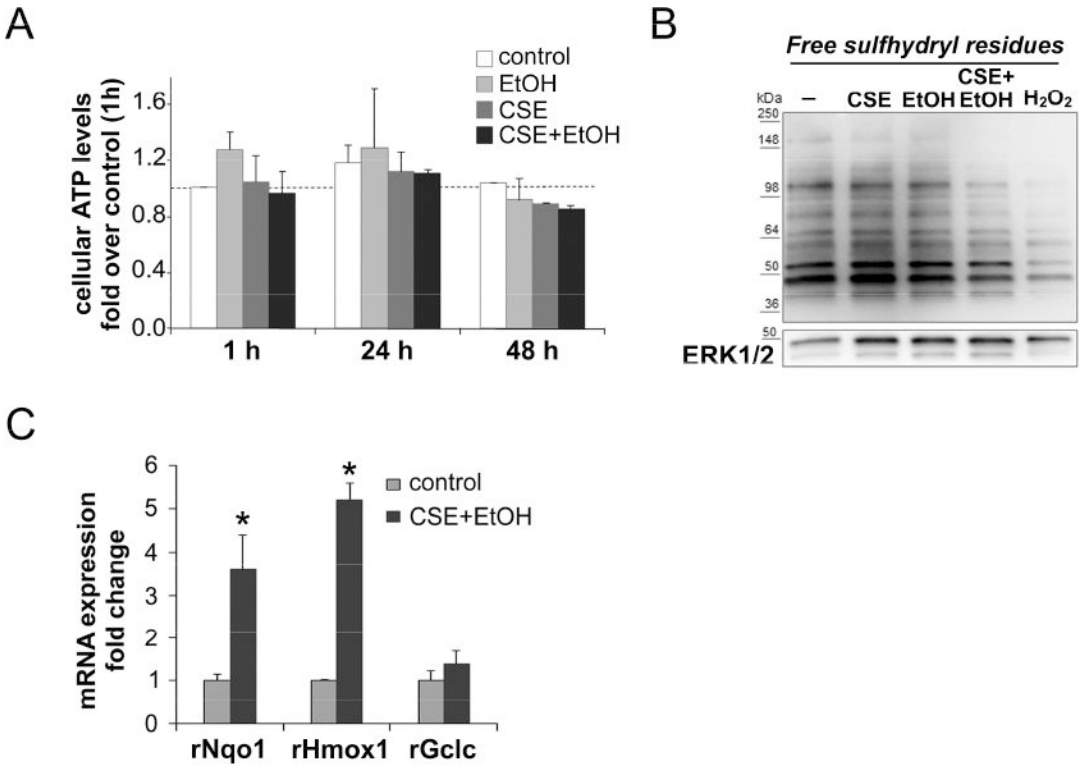

Figure 4. Ethanol and smoking induce oxidative stress and have minor effects on cellular ATP levels

(A) AR42J cells were treated for the indicated times with 50 mM EtOH or 40 μg/ml CSE alone or in combination. Total cellular ATP levels were measured by bioluminescence assay. Graph shows mean±SEM, n=3. (B) CSE+EtOH induce biological oxidation of sulfhydryl residues. AR42J cells were left untreated or treated with 50 mM EtOH, 40 μg/ml CSE or CSE+EtOH for 48 h. Cells exposed to 1 mM H_2O_2 for 30 min were used as positive controls. Cell lysates were incubated with BIAM (0.5 mM), which reacts with free sulfhydryl residues on proteins. Immunoblots show biotinylated protein levels detected by streptavidin-HRP antibody. ERK1/2 was used as loading control. Data are representative of three independent experiments. (C) AR42J cells were treated with CSE+EtOH or vehicle for 48 h. Expression levels of the rat oxidative stress response genes rNqo1, rHmox1 and rGclc were measured by qPCR. Graph shows mean±SEM, n=3; * P<.05 vs. control.

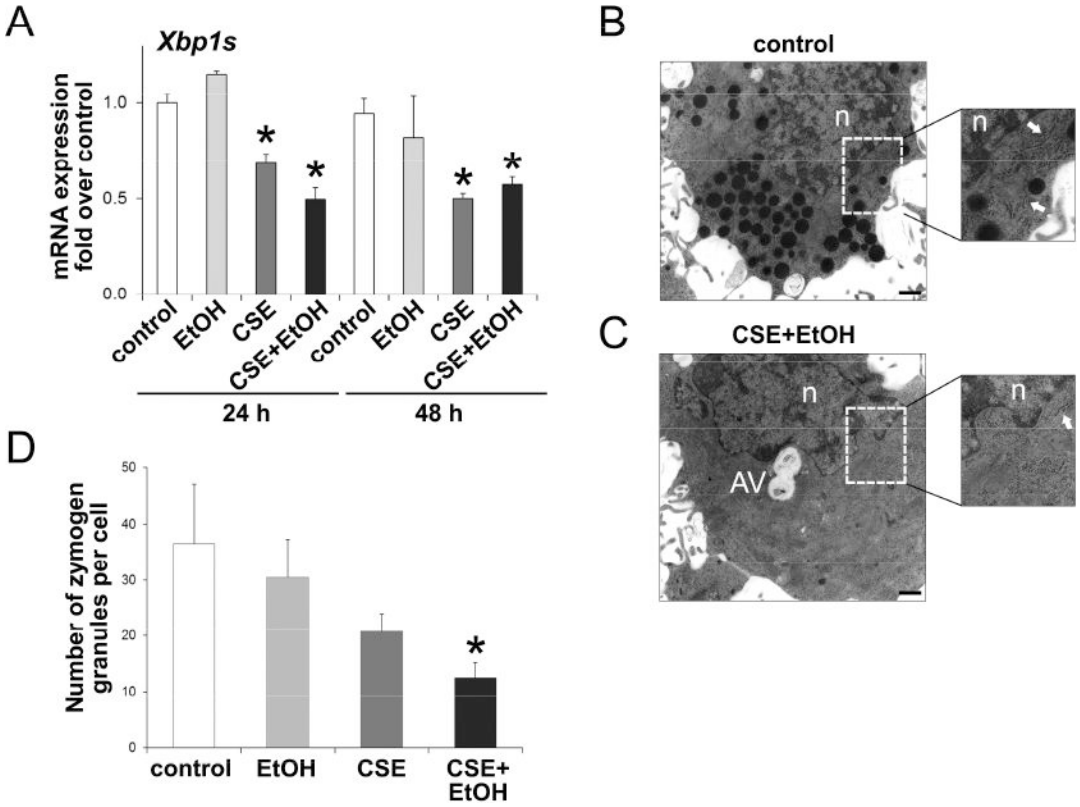

Figure 5. CSE markedly reduces XBP1s expression, the ER network and the number of zymogen granules in acinar cells

(A) Expression levels of XBP1s were measured in AR42J cells treated with 50 mM EtOH and/or 40 μg/ml CSE for the indicated times. Data represents mean±SEM, n=3; * *P<.05 vs. control.* (B-C) Electron micrographs from AR42J cells left untreated (control, panel B) or treated with CSE+EtOH (panel C). Control cells display normal ultrastructure with high numbers of zymogen granules (Z) and bundles of rough endoplasmic reticulum (ER) that can be visualized at higher magnification in the right insert (white arrows). CSE+EtOH treated cells (panel C) display low density of zymogen granules, sparse ER (insert at right) and occasional autophagic vacuoles (AV). n, nucleus; Bars, 0.5 μm. (D). Graph shows number of zymogen granules per cell measured in electron micrographs from AR42J cells treated as indicated (mean±SEM, n=20-25 cells). *P<.05 vs. control.

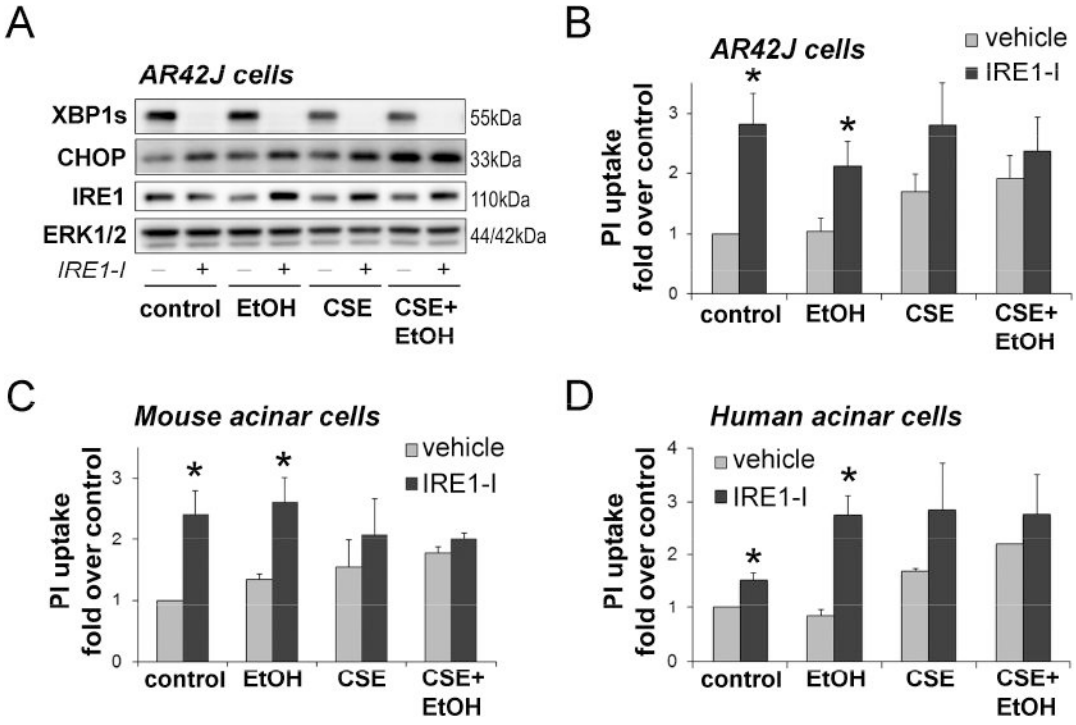

Figure 6. Inhibition of XBP1s formation recapitulates acinar cell death induced by CSE and ethanol

(A and B) AR42J cells were treated with 50 mM EtOH and/or 40 μg/ml CSE for 48 h in the presence of the IRE1-RNase inhibitor (IRE1-I) or vehicle. (A) Immunoblot shows protein levels of XBP1s, CHOP, IRE1 and ERK1/2. (B) Graph shows cell death in AR42J cells assessed by PI uptake. (C and D) Graphs illustrate cell death in mouse and human acinar cells treated for 24 h in the same conditions as AR42J cells in panels A and B. Data in graphs are mean±SEM, n=3; *$P<.05$ *vs. control*.

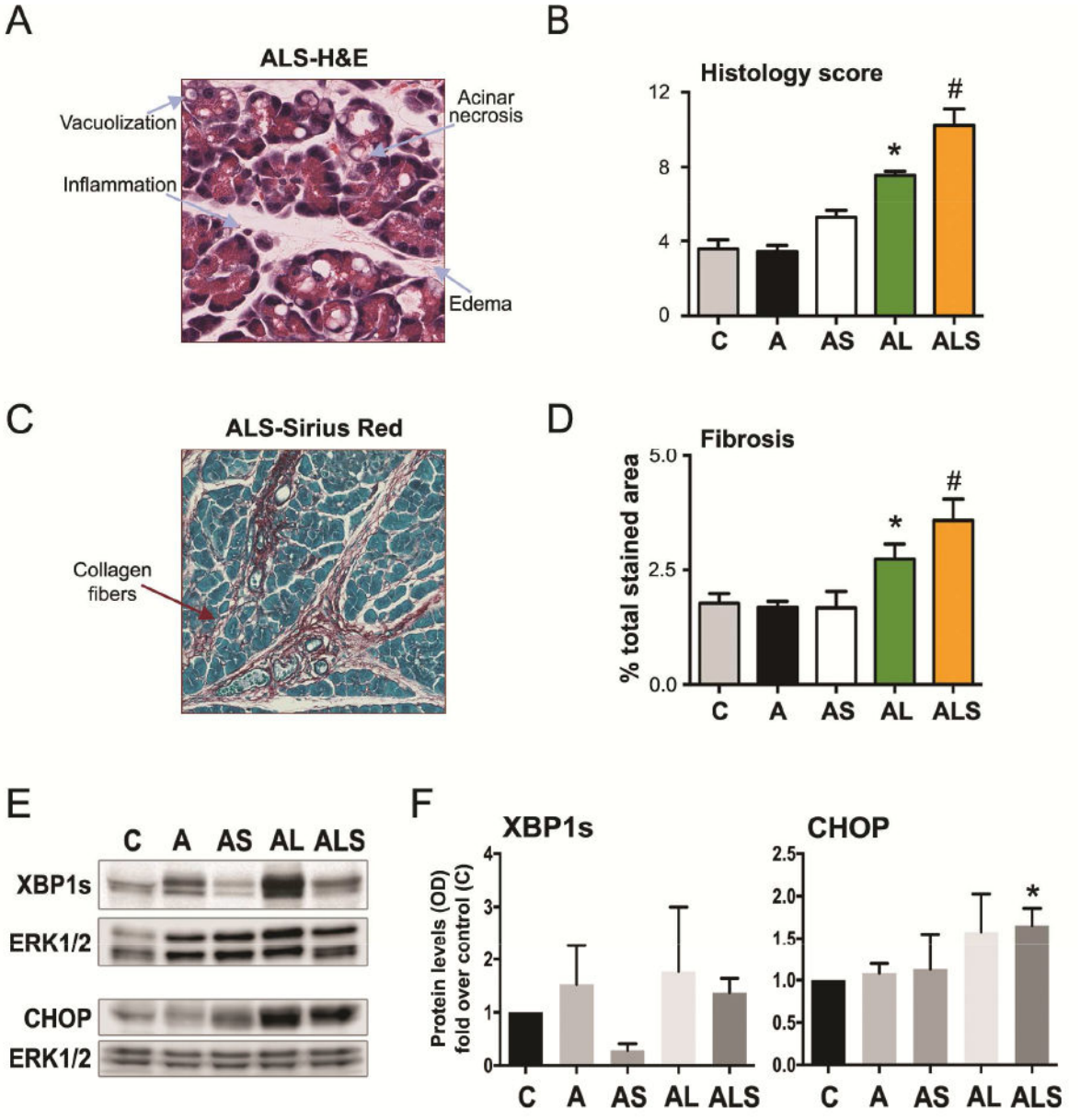

Figure 7. Smoking worsens pancreatitis induced by alcohol and LPS

Rats fed control or ethanol-containing diet were exposed to cigarette smoke and treated with LPS to induce pancreatitis as indicated in Supplementary Methods. Figure shows data for the following groups: C, control diet; A, alcohol diet; AS, alcohol+smoke exposure; AL, alcohol+LPS; ALS, alcohol+LPS+smoke exposure. (A) and (C), representative photomicrographs depicting pancreatic injury (A) and fibrosis (C; collagens, Sirius red staining) in the ALS group. Graphs in (B) and (D) show quantitative analyses (mean±SEM, n=3) for pancreatic injury and Sirius red staining; *P<.05 vs. A; #<.05 vs. ALS. (E) Representative immunoblots show pancreatic protein levels of the indicated UPR markers and ERK1/2 as loading control. Each lane shows data from an individual rat. (F) Graphs

show quantitation of optical density of XBP1s and CHOP bands in immunoblots relative to those of ERK1/2. Data are mean±SEM, n=3 rats/group.