Ethanol and tobacco smoke increase hepatic steatosis and hypoxia in the hypercholesterolemic apoE-/- mouse: implications for a "multi-hit" hypothesis of fatty liver disease

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Abstract

While epidemiologic studies indicate that combined exposures to cigarette smoke and alcohol increase risk and severity of liver diseases, the molecular mechanisms responsible for hepatotoxicity are unknown. Similarly, emerging evidence indicates a linkage among hepatic steatosis and cardiovascular disease. Herein, we hypothesize that combined exposure to alcohol and environmental tobacco smoke (ETS) on a hypercholesterolemic background increase liver injury through oxidative/ nitrative stress, hypoxia, and mitochondrial damage. To test this, male apoE-/- mice were exposed to an ethanol-containing diet, ETS alone, or a combination, and histology and functional endpoints were compared to filtered air, ethanol-naïve controls. While ethanol consumption induced a mild steatosis, combined exposure to ethanol + ETS resulted in increased hepatic steatosis, inflammation, alpha smooth muscle actin, and collagen. Exposure to ethanol + ETS induced the largest increase on CYP2E1 and iNOS protein, as well as increased 3-nitrotyrosine, mtDNA damage, and decreased cytochrome *c* oxidase protein compared to all other groups. Similarly, the largest increase in HIF1α expression was observed in the ethanol + ETS group indicating enhanced hypoxia. These studies demonstrate that ETS increases alcohol-dependent steatosis and hypoxic stress. Therefore, ETS may be a key environmental "hit" that accelerates and exacerbates alcoholic liver disease in hypercholesterolemic apoE-/- mice.

Keywords

liver; alcohol; steatosis; mitochondria; cigarette smoke; hypercholesterolemia; oxidative stress; hypoxia; cytochrome *c* oxidase; blue native gel electrophoresis

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Introduction

Excessive consumption of alcohol (i.e. ethanol) causes liver disease by a combination of pathologic mechanisms including oxidative/nitrative stress, inflammation, redox alterations, and mitochondrial damage [1,2]. During the early stages of alcoholic liver disease gut-derived endotoxin activates Kupffer cells, the resident hepatic macrophages, which release cytokines and reactive oxygen/nitrogen species (ROS/RNS) that negatively affect hepatocyte function [3,4] particularly at the level of the mitochondrion. This alcohol-dependent depression in mitochondrial function is proposed to contribute to disease progression and severity via increased oxidative stress and bioenergetic defects leading to hepatocyte cell death [5]. However, it has been proposed that additional exposures or stressors are necessary for severe liver pathology to occur in the chronic alcohol consumer [6]. This concept is reinforced by statistics showing that only 25% of heavy drinkers develop alcoholic steatohepatitis with even fewer progressing to cirrhosis [7]. These observations have lead to a new hypothesis in the hepatology field that genetic, viral, metabolic, and/or environmental stressors or "hits" accelerate the progression from simple fatty liver (steatosis) to more severe liver pathologies including steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [8].

While the direct hepatotoxic effects of alcohol have been extensively studied, the impacts of metabolic and/or environmental stressors or "hits" on those processes that lead to alcohol-induced hepatotoxicity remain undefined. Examples of these "hits" include metabolic stressors such as hyperglycemia, hypertriglyceridemia, and hypercholesterolemia that are associated with cardiovascular diseases and diabetes, whereas environmental stressors may include exposures to dietary factors, industrial pollutants, and tobacco smoke. It is estimated that alcohol and cigarette smoke exposures are the underlying causes in 35% and 50% of all deaths, respectively [9]. Moreover, while "second-hand" or environmental tobacco smoke (ETS), a widespread environmental toxicant, is a known risk factor for heart disease [10], the hepatotoxic potential of direct and indirect cigarette smoke exposure is only now being recognized [11]. Seminal work by Klatsky and colleagues showed that cigarette smoke exposure promotes alcoholic cirrhosis, increases certain cancers, and possibly increases mortality in heavy alcohol drinkers [12,13]. Recent epidemiologic studies also suggest that cigarette smoke exposure accelerates a number of chronic liver diseases including hepatitis C and primary biliary cirrhosis, and increases the risk for hepatocellular carcinoma [14-18]. Importantly, obesity has been identified as a risk factor for alcoholic liver disease [19,20] and alcohol, tobacco smoke, and obesity have been shown to be synergistic risk factors for hepatocellular carcinoma [21]. Taken together, these studies clearly support the hypothesis that tobacco smoke and other environmental "hits" may worsen the pathologic effects of alcohol in the liver, especially in obese individuals and those suffering from pre-existing illnesses like cardiovascular disease.

Although findings from epidemiologic studies demonstrate a correlative association between ethanol and ETS to worsen liver disease, the molecular mechanisms responsible for the interactive effects of ETS and ethanol to aggravate liver pathology are not known. Emerging evidence from the cardiovascular field strongly implicates oxidative stress and mitochondrial dysfunction with altered nitric oxide (NO) bioavailability in the disease process. For example, cigarette smoke damages the microvasculature via endothelial cell dysfunction and smooth muscle cell proliferation leading to impaired NO signaling and tissue hypoxia [22]. Rats exposed to ETS from only two cigarettes per day for two months have severely damaged myocardial oxidative phosphorylation function following ischemia-reperfusion [23]. Furthermore, ETS exposure in hypercholesterolemic mice causes significant aortic mitochondrial DNA (mtDNA) damage, increased tyrosine nitration, and inactivation of manganese superoxide dismutase when compared to hypercholesterolemia alone [24,25]. These studies also demonstrate that when combined with hypercholesterolemia, ETS

accelerates both mitochondrial damage and atherosclerosis [25]. Importantly, our laboratories have demonstrated that combined exposure to ethanol and ETS exacerbates atherosclerosis, which is accompanied by increased mtDNA damage and oxidant stress in heart and aorta when compared to hypercholesterolemic mice exposed to ethanol or ETS alone [26]. Due to the large body of literature advancing the cardio-protective benefits provided by mild-to-moderate alcohol consumption [27,28], the study by Cakir and colleagues highlights the importance of considering the potential negative impacts of alcohol on the cardiovascular system when combined with known cardiovascular risk factors like hypercholesterolemia and ETS [26]. Indeed, the cardio-protective effects of alcohol have recently been questioned in several excellent review articles [29-31].

Because exposure to ETS is widespread and often occurs concurrently with alcohol consumption, investigations of their combined toxicities on the progression and severity of alcoholic liver disease are warranted and in the interest of public health. The importance of this is further supported because many of the responses elicited by cardiovascular risk factors like ETS and hypercholesterolemia are identical to those found in liver following chronic alcohol consumption. Indeed, there are overlapping impacts of chronic ethanol consumption, ETS, and hypercholesterolemia on mitochondrial function, which involve increased production of ROS/RNS and accumulation of mtDNA damage. Given this, and the likelihood that the chronic alcohol consumer is also likely to suffer from conditions of the cardiometabolic syndrome, it is crucial to begin to define the molecular mechanisms through which ETS and cardiovascular risk factors may accelerate alcoholic liver disease. To this end, several laboratories are using dyslipidemic mice models traditionally reserved for cardiovascular disease investigations to inform studies on the pathobiologic mechanisms underlying fatty liver disease. One approach has been to use hypercholesterolemic mouse models like the apoE$^{-/-}$ especially because it is known that apoE lipoprotein may play a key role in hepatic lipid metabolism and β-oxidation [32]. Specifically, studies show that triglyceride-very low density lipoprotein (VLDL) secretion is impaired in livers of ldlr$^{-/-}$ and apoE$^{-/-}$ mice and leads to development of fatty liver when mice are fed a Western diet [33-35]. These findings suggest that the apoE$^{-/-}$ mouse might be a novel model to study mechanisms of fatty liver disease from both alcohol and non-alcohol mediated sources, e.g. from high fat or high carbohydrate diets. Therefore, the current study builds upon our previous work demonstrating increased cardiovascular damage from combined exposures to ethanol and ETS [26] and was conducted to test the hypothesis that concurrent ethanol and ETS exposure on a dyslipidemic background significantly increase liver steatosis and injury through increased oxidative/nitrative stress, hypoxia, and mitochondrial damage.

Materials and methods

Animals

Male apoE$^{-/-}$ mice (C57BL/6 background, 6 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate for 1 wk before beginning liquid diets. The apoE$^{-/-}$ mouse lacks apolipoprotein E, which is required for receptor mediated low density lipoprotein (LDL) cholesterol uptake from the blood. Consequently, mice are dyslipidemic with elevated levels of serum VLDL, LDL, cholesterol, and triglycerides, which predispose mice to develop atherosclerotic lesions similar to humans [36]. Mice were handled in accordance with guidelines outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23 revised 1985) and the research protocol for these studies was approved by the institutional animal care and use committee.

Chronic ethanol consumption and ETS exposures

Animal exposures for these studies were performed at the Institute of Toxicology and Environmental Health inhalation facilities (University of California, Davis) as described previously [26]. Mice were fed nutritionally adequate control and ethanol-containing liquid diets formulated by Bio-Serv (Frenchtown, NJ). The ethanol-containing diet provided 28.8% of the total daily caloric intake as ethanol, 35% as fat, 18% as protein, and 18.2% as carbohydrate (maltose-dextrin). Control animals were pair-fed identical diets except that ethanol calories were replaced isocalorically with carbohydrate [37]. The ethanol concentration in the diet was increased from 0 to 28.8% of total daily caloric intake over a 9 day period and then maintained at the highest dose, 4% (w/v) ethanol, for the duration of the study to achieve blood ethanol concentrations of approximately 10 mM, which are in agreement with previously reported values [37]. ETS exposures and filtered air exposures began on day 14 after mice were acclimated to the 4% (w/v) ethanol-containing diet. Mice were exposed to ETS (10 mg/m^3 total suspended particulate, TSP) or filtered air 5 hr per day, 5 days a week (Mon-Fri) for 4 additional weeks [26]. The concentration of carbon monoxide and nicotine at this ETS dose averaged 32.6 ± 5.6 ppm and 1.41 ± 0.2 mg/m^3, respectively, throughout the 4 week exposure period. As noted previously [26] this dose of ETS is well below that reported in similar studies in rodents [38] and mimics that of physiologically relevant doses experienced by humans exposed to passive and/or active cigarette smoking [39]. In summary, there were 4 treatment groups for this study: 1) control diet + filtered air; 2) ethanol diet + filtered air; 3) control diet + ETS; and 4) ethanol diet + ETS.

Liver histopathology and Sirius red staining

Formalin-fixed liver samples were processed for hematoxylin-eosin staining and evaluated for steatosis, hepatocyte ballooning, and Mallory bodies by two hepatologists that were blinded to the experimental conditions [40]. The degree of steatosis (% of hepatocytes containing fat), the presence of intra-acinar (i.e. lobular) inflammation, and hepatocyte ballooning were used to define the severity of pathology. Steatosis was assessed by determining the percentage of hepatocytes containing fat droplets as 0 (< 5%); 1+ (6-25%); 2+ (26-50%); and 3+ (51-75%). Intra-acinar inflammation was scored as the presence of inflammatory cell aggregates: absence of inflammation was scored 0, 1 foci was scored 1, and 2-4 foci were scored 2 per 10X objective.

Collagen fibers were visualized in liver using Sirius red staining as described in [41]. Briefly, formalin-fixed liver sections were de-paraffinized and rehydrated by standard methods and then stained with 0.1% (w/v) Sirius red (Direct Red 80, Sigma-Aldrich, St. Louis, MO) in a saturated aqueous solution of picric acid for 60 min. The red-stained collagen fibers were quantified using the ImageJ software package (National Institutes of Health, Bethesda, MD) and expressed as percent area [41].

Immunoblot analyses for alpha smooth muscle actin, cytochrome P450 2E1 (CYP2E1), and inducible nitric oxide synthase (iNOS)

Immunoblots were performed by loading 20-100 μg of whole liver homogenate onto SDS-PAGE gels with proteins electroblotted onto nitrocellulose membranes for incubations with primary antibodies. Levels of iNOS protein were detected using a 1:5,000 dilution of antibody (Chemicon International, Temucula, CA). CYP2E1 protein was detected using a 1:1,000 dilution (Chemicon International) and alpha smooth muscle actin protein was detected using a 1:1,000 dilution of antibody (Dako, Carpinteria, CA). After incubation of membranes with appropriate HRP-conjugated secondary antibodies (Sigma Chemical Company, St. Louis), proteins were visualized using chemiluminescence detection methods and the intensity of immunoreactive protein bands were quantified using NIH Image (Bethesda, MD). The protein concentration loaded onto gels was within the linear range of the chemiluminescence system used for all proteins.

Immunostaining for hepatic 3-nitrotyrosine (3-NT), hypoxia inducible factor 1alpha (HIF1α), and alpha smooth muscle actin

A hallmark feature of RNS generation is nitration of tyrosine residues forming 3-NT residues, which can be detected by immunohistochemistry as described in [37]. Formalin-fixed liver sections were de-pariffinized in xylene and rehydrated through incubations in graded ethanol concentrations. Tissue sections were subjected to antigen retrieval in 10 mM citrate buffer, pH 6.0, endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 10 min, and blocking was performed for 1 hr at room temperature with 10% goat serum diluted in a 1% (w/v) BSA-containing Tris-buffered saline with Tween-20 (TBS-T) solution. Sections were incubated for 2 hr at room temperature in a humidified chamber with rabbit polyclonal 3-NT antiserum at a 1:150 dilution. Following incubation, sections were washed, incubated with secondary antibody at a 1:200 dilution, and bound antibody was visualized with diaminobenzidine.

Hypoxia was verified by assessing the response of the hypoxia-sensitive protein HIF1α essentially as described above with minor modifications. Liver sections were subjected to antigen retrieval and blocking, followed by an overnight incubation at 4°C in a humidified chamber with anti-HIF1α antiserum (Lab Vision Corp., CA) at a 1:50 dilution. Following incubation, sections were washed in a 0.025% (w/v) Triton X-100, TBS solution and incubated with the secondary antibody, Alexa Fluor® 350 conjugated goat anti-mouse (Invitrogen, Eugene, OR) at a 1:50 dilution for 1 hr. Sections were washed with TBS and counterstained with DAPI (8 µg/mL, Sigma, St. Louis, MO) for 10 min to visualize nuclei.

Immunostaining for alpha smooth muscle actin was performed by standard methods [42]. After liver sections were de-parrifinized, rehydrated, incubated with 3% H_2O_2, and washed, they were blocked with 10% (w/v) BSA/TBS-T. Liver sections were incubated with alpha smooth muscle actin antiserum at a 1:800 dilution. After washings, liver sections were incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution followed by diaminobenzidine to visualize alpha smooth muscle actin. Smooth muscle actin and 3-NT were visualized as brown staining with brown area intensity quantified using either ImageJ software package (National Institutes of Health, Bethesda, MD) or Simple PCI software (Hamamatsu Corporation, Sewickley, PA), respectively. For HIF1α, images were taken and analyzed using a Leica fluorescent microscope with IPLAB Spectrum (BD Biosciences Bioimaging, Scanalytics, Rockville, MD) and the intensity of fluorescence quantified using Simple PCI software.

Blue native gel electrophoresis (BN-PAGE)

Mitochondria were isolated by differential centrifugation and BN-PAGE was performed as described in [40,43]. For BN-PAGE, mitochondrial protein (1 mg) was suspended in extraction buffer (0.75 M aminocaproic acid, 50 mM Bis-Tris, and 1% (w/v) N-docecyl-β-D maltoside) and incubated on ice to release the intact respiratory complexes from the inner membrane. After incubation, Coomassie Blue G-250 was added and mitochondrial extracts (250 µg) were loaded on to non-denaturing gels to separate the individual protein complexes [44]. Gels were stained and complex densities were quantified using Scion Image Beta 4.01 (Scion Image Corp.) as described in [43]. There was no difference in mitochondrial protein yield and citrate synthase activities among groups [control + filtered air, 0.506 ± 0.021; ethanol + filtered air, 0.474 ± 0.042; control + ETS, 0.525 ± 0.027; ethanol + ETS, 0.545 ± 0.022 µmol/min/mg protein, p=0.41]. This is important because it demonstrates that the high levels of fat in liver of the ethanol + ETS group had no effect on isolation, overall content, and purity of mitochondria used for BN-PAGE. Cytochrome *c* oxidase activity was measured using a standard spectrophotometric method that monitors the oxidation of ferrocytochrome *c* at 550

nm [45] and oxidase activities were normalized to citrate synthase activities [46] for each individual mitochondrial sample.

Quantitative polymerase chain reaction (QPCR) for mtDNA damage

Genomic DNA was extracted from flash-frozen liver tissue, quantified using PicoGreen (Molecular Probes, Eugene, OR), and QPCR performed as described previously [40,43]. The principle of this method is that DNA lesions block the rTth polymerase, which results in decreased amplification of the gene target of interest, i.e. mitochondrial genome. Sensitivity of the assay is increased via amplification of large targets thereby increasing the probability of encountering a DNA lesion. A 16,059 bp QPCR product that includes all but 236 bp of the NADH5/6 genes in the mouse mtDNA genome was amplified using a Gene AMP PCR system 2400 with Accuprime polymerase and a specified primer set [25]. Copy number differences in mtDNA were normalized using a short QPCR reaction (base pairs 12281-13361 of the mtDNA), which yields products directly related to gene copy numbers. Mitochondrial DNA damage was quantified by comparing the relative efficiency of amplification of large (>15 kb) fragments of DNA and normalizing this to gene copy numbers by the amplification of smaller (<250 bp) fragments, which have a statistically negligible likelihood of containing damaged bases. DNA lesion frequencies were calculated as described in [47].

Statistical Analyses

Data represent the mean ± SEM for 3-6 pairs of animals in each group. Significant differences between treatments were obtained using paired and unpaired t-tests and two-factor ANOVA, $p < 0.05$. The Mann-Whitney rank sum test was used for pathology analyses.

Results

Male apoE$^{-/-}$ mice were pair-fed either a control or ethanol-containing liquid diet for 6 weeks with ETS or filtered air exposure during the last 4 weeks of the experimental study as described previously [26]. Mice were exposed to ETS at 10 mg/m^3 total suspended particulate 5 hr per day/5 days per week; an exposure that is well below those in published reports on ETS exposure in rodents [48]. Ethanol and ETS exposure had no effect on body weight [control + filtered air, 24.3 ± 0.7; ethanol + filtered air, 23.7 ± 1.3; control + ETS, 24.0 ± 1.73; and ethanol + ETS, 27.3 ± 1.5 g, p=0.18]. Similarly, there was no difference in ethanol consumption between the ETS and filtered air exposure groups throughout the feeding period [ethanol + filtered air, 12.3 ± 0.3 and ethanol + ETS, 13.6 ± 0.3 mL of diet/day, p=0.6]. These results of ethanol consumption are similar to those previously seen in our laboratories when using mice [37] and demonstrate that ETS exposure has no effect on ethanol consumption.

Histologic examination of livers showed a large accumulation of macrovesicular, as well as microvesicular fat in the livers of apoE$^{-/-}$ mice exposed to ETS and fed an ethanol-containing diet when compared to all other experimental groups (Figure 1A, panel d). Livers from the ethanol alone group show only a mild steatosis (Figure 1A, panel b), which agrees with our previous studies using wild-type and iNOS$^{-/-}$ mice fed the same ethanol-containing diet [37]. Quantification of steatosis demonstrates that while only 5% of hepatocytes contained fat droplets in the ethanol alone group, 50% of hepatocytes across all zones of the liver lobule contained fat in the ethanol + ETS group (Figure 1B). Moreover, livers from both control groups (ETS alone or filtered air) show no signs of fat accumulation and exhibited no pathological changes (Figure 1A, panels a and c), consistent with previous studies using this same corn oil based-high fat diet in mice [37]. Hepatocyte ballooning was observed in 1 of 3 ethanol exposed mice and in 1 of 3 ethanol + ETS treated mice, whereas hepatocyte ballooning was absent in both control groups.

This large increase in steatosis in the ethanol + ETS group was accompanied by increased inflammatory foci and necrosis. Figure 1D shows increased neutrophils and lymphocytes within the liver parenchyma from animals exposed to both ethanol and ETS (see arrows). While there was increased inflammation in the ethanol + ETS group as compared to ethanol alone this difference was not statistically significant (Figure 1C). Accompanying the histologic presence of steatosis, co-exposure to ethanol + ETS also resulted in a significant increase in the liver weight and liver/body weight ratio as compared to ethanol and ETS only groups (Figure 2) indicating liver injury. ETS exposure alone had no effect on these two parameters when compared to the control diet only group.

Previous studies have shown that hypoxia is induced in liver by both acute and chronic ethanol consumption and contributes to hepatotoxicity [49,50]. To determine whether ETS enhanced ethanol-mediated hypoxia, HIF1α expression was measured by immunofluorescence. Figure 3 shows a significant increase in HIF1α staining in liver from ethanol + filtered air mice (Figure 3A, panel b) when compared to their corresponding control (Figure 3A, panel a). There was no difference in HIF1α expression in mice fed control diets and those exposed to ETS only (Figure 3A, compare panels a and c). Interestingly, there was a significant increase in HIF1α expression in liver of mice co-exposed to ethanol and ETS (Figure 3A, panel d) when compared to the ethanol only group (Figure 3A, panel b). Quantification of HIF1α expression is presented in Figure 3B.

Because these findings suggested acceleration in liver injury by combined exposures to ethanol and ETS, the levels of alpha smooth muscle actin were determined as an early marker of fibrogenesis. Immunoblot analysis demonstrates that combined exposure to ethanol + ETS increased the level of alpha smooth muscle actin in liver by 65% when compared to the ETS alone group and 40% when compared to the ethanol alone group (Figure 4A). Ethanol consumption alone increased the levels of alpha smooth muscle actin by 50% compared to the control + filtered air; however, this increase was not statistically significant (p=0.078). ETS had no effect on the levels of alpha smooth muscle actin in control fed animals. The ethanol and ETS-associated effects on alpha smooth muscle actin were also confirmed and visualized with immunohistochemistry (Figure 4B) with increased staining around veins and in tissue (Figure 4B, panel d). In addition, livers from ethanol + ETS group showed increased staining for collagen compared to all treatment groups (Figure 5). Increased collagen staining (i.e. faint, red fibers) was observed radiating out from the central vein regions in livers from the ethanol + ETS group (see arrows, Figure 5A, panel d) whereas red staining is only seen around vessels in the other treatment groups. Quantification of Sirius red staining is presented in Figure 5B.

There are several potential sources of ROS/RNS in hepatocytes, which are induced or altered by chronic ethanol consumption resulting in increased production of oxidants. The data shown in Figure 6 confirms results demonstrating induction of hepatic CYP2E1 protein in response to chronic ethanol consumption [40]. More important, is the observation that the combination of ethanol and ETS resulted in a much larger increase in CYP2E1 protein levels when compared to the ethanol alone group (Figure 6).

Increased expression of iNOS has also been associated with chronic alcohol-dependent oxidative/nitrative stress and inflammation [37,51]; however, the impact of ETS on hepatic iNOS expression is not known. While there was a modest ethanol associated increase in iNOS protein, the combination of ethanol and ETS significantly increased hepatic iNOS protein levels when compared to either ethanol or ETS alone (Figure 7). These results are in agreement with those showing increased heart iNOS transcripts and protein following ethanol and ETS exposure [26]. Similarly, we assessed the effect of ethanol and ETS on protein nitration by measuring the levels of 3-NT in liver tissue as described previously [37]. As shown in Figure 8, little to no 3-NT was observed in the liver of mice exposed to the control diet only (Figure

8A, panel a), with increased 3-NT in the ETS alone (panel c) and ethanol alone (panel b) groups. In contrast, there was a large increase in 3-NT staining in the ethanol + ETS group (panel d), which was significantly increased over all treatments (Figure 8B). These results, in combination with those presented for iNOS, suggest that simultaneous exposure to ethanol and ETS causes a significant increase in RNS production and potential nitrative stress in liver.

Based on results indicating increased oxidative and nitrative stress in liver of mice exposed to ethanol and/or ETS, it was predicted that there would be increased mitochondrial damage in response to both toxicant exposures. To examine the effect of ethanol and ETS on mitochondria, mtDNA damage was assessed using QPCR. As shown in Figure 9, mtDNA damage progressively increased from low levels of mtDNA damage in the ethanol alone group to a substantially higher level in the ETS and ethanol + ETS exposure groups. Thus, through damaging the mtDNA it is hypothesized that combined exposure to ethanol and ETS will increase mitochondrial dysfunction and worsen liver injury.

To assess the effect of ethanol, ETS, and ethanol + ETS on the mitochondrial oxidative phosphorylation system, BN-PAGE was used. BN-PAGE is a specialized form of gel electrophoresis that is particularly well-suited for the separation and analysis of the large, hydrophobic respiratory complexes that reside in the mitochondrial inner membrane and comprise the oxidative phosphorylation system [52]. Figure 10A shows a representative BN-PAGE separation of the five oxidative phosphorylation system complexes from each treatment group. The complexes are separated intact on a non-denaturing gel, arranged vertically based on molecular weight, and are visualized as large protein bands (Figure 10A, arrows). Quantification of the density of each complex revealed modest decreases in protein content in response to ethanol or ethanol + ETS for complexes I, II, III, and V; however, none of these differences were statistically significant (Figure 10B). However, there was a significant decrease in the content of cytochrome *c* oxidase (i.e. complex IV) in the ethanol + ETS group when compared to the ethanol only group (p=0.022). Similarly, two-factor ANOVA analyses showed a statistically significant effect of ETS on complex IV content compared to filtered air groups (p=0.015). Cytochrome *c* oxidase activity was also decreased in the ethanol + ETS group compared to the ethanol only group; however, this difference was not statistically significant [control + filtered air, 0.093 ± 0.02; ethanol + filtered air, 0.086 ± 0.02; control + ETS, 0.091 ± 0.03; ethanol + ETS, 0.071 ± 0.015 U/units citrate synthase, p=0.19, ethanol only compared to ethanol + ETS].

Discussion

Studies designed to investigate the combined toxicities of ethanol and cigarette smoke on liver pathogenesis have largely relied on ethanol exposure in combination with nicotine injections, which have shown decreased antioxidants, increased lipid peroxidation, and increased CYP2E1 levels [53-55]. Despite these efforts, it has been difficult to investigate the physiologic and mechanistic mechanisms through which cigarette smoke and especially "second-hand" exposure (i.e. ETS), worsen liver disease in the ethanol consumer. To address this issue, Gentry-Nielsen and colleagues made significant progress by developing a physiologically relevant rat model of combined ethanol and cigarette smoke exposure [38]. Using this model, they observed alcohol-dependent defects in liver function, decreased asialoglycoprotein receptor binding, and alterations in blood cholesterol levels with no additional metabolic disturbances observed when rats were co-exposed to cigarette smoke [38]. Based on this [38] and the knowledge that fatty liver disease is now associated with cardiovascular disease risk [56,57], we propose that experimental models of dyslipidemia and atherosclerosis, like the apoE$^{-/-}$ hypercholesterolemic mouse, may be important tools to better understand mechanisms of liver disease from combined exposures to ethanol and other environmental toxicants. This concept is strengthened by reports that hypercholesterolemic ldlr$^{-/-}$ and apoE$^{-/-}$ mice develop

steatohepatitis when fed a high fat, Western diet supplemented with cholesterol [33-35]. While the mechanism for increased steatosis in these hypercholesterolemic mouse models is not clear, it has been suggested that in addition to its role in lipoprotein clearance, apoE protein may function in VLDL assembly and secretion [33]. As a consequence, apoE deficiency may contribute, in part, to the development of steatosis due to reduced VLDL-triglyceride secretion, which is not related to defects in triglyceride turnover, diacylglycerol acyltransferase activity, or microsomal triglyceride transfer protein levels [58].

Additionally, impaired VLDL secretion may not be the sole cause for steatosis in the apoE$^{-/-}$ mouse. For example, changes in the expression of β-oxidation and lipid metabolism proteins may underlie fat accumulation as shown in mice expressing the mutant APOE*3 Leiden isoform that were fed a high fat/cholesterol-containing diet [32]. In humans, the mutant APOE*Leiden leads to dysbetalipoproteinemia [59] and mice expressing this transgene develop hyperlipidemia due to impaired binding of mutant-remnant lipoproteins to the LDL receptor [60]. To date, it is unclear whether there is a link between hypercholesterolemia (i.e. APOE genotype) and the incidence and severity of hepatic steatosis associated with the cardiometabolic syndrome or excessive alcohol intake. However, two recent studies indicate an association between APOE polymorphisms in alcohol and nonalcoholic steatohepatitis. Specifically, Panduro and colleagues found an association between hypertriglyceridemia and the APOE*2 allele with early onset alcoholic cirrhosis [61], whereas the APOE*3 allele was overrepresented in a group of nonalcoholic steatohepatitis patients [62]. This, taken together with data presented herein demonstrating a large increase in steatosis in apoE$^{-/-}$ mice exposed to ethanol and ETS, supports the concept that genetically determined stressors like hypercholesterolemia may be important additional metabolic "hits" that should be considered in studies of the molecular events involved in liver disease pathogenesis. To clarify this, future studies using wild-type mice exposed to ethanol and ETS are warranted to shed light on the precise role of hypercholesterolemia in this "multi-hit" model of fatty liver disease. These studies are critical to determining whether or not a dyslipidemic genetic background is required for the steatosis-enhancing properties of ETS in a model of alcoholic liver disease. It is possible that ETS may not enhance alcohol-dependent steatosis in the absence of dyslipidemia.

Using well-established models of ETS exposure and chronic ethanol consumption, we observed that steatosis was significantly increased by simultaneous exposures to ETS and ethanol, which was accompanied by increased liver weight and liver/body weight ratio; additional measures of liver injury. Combined exposure to ETS and ethanol also increased the number of inflammatory foci in liver, as well as alpha smooth muscle actin levels and collagen; early indicators of fibrogenesis. As discussed earlier, the metabolic defects associated with hypercholesterolemia may increase fat accumulation in liver through alterations in lipoprotein and triglyceride processing [58], thereby predisposing liver to increased damage when exposed to "hits" like ethanol and ETS. It should be pointed that previous studies showing increased fat in untreated apoE$^{-/-}$ mice were conducted using Western diets high in butter fat or palm oil with varying concentrations of added cholesterol [33,34]. In contrast, the lack of overt steatosis in the untreated control group in this current study may be due to differences in dietary fat type. For example, the diet used herein was a corn oil based (largely linoleate) and contained no added cholesterol. It is predicted that the inclusion of cholesterol in this diet may have enhanced steatosis in all groups. Previously, we demonstrated that chronic ethanol consumption increased total plasma cholesterol in apoE$^{-/-}$ mice, which when combined with ETS exposure was elevated to much higher levels [26]. Important to the current study, is the report from Mari et al [63] demonstrating that cholesterol loading of liver and mitochondria increases tumor necrosis factor α-dependent liver injury due to mitochondrial glutathione depletion, thereby sensitizing the liver to oxidative and nitrative stress. Consequently, increased cholesterol is proposed to accelerate the progression from simple steatosis to more severe liver pathologies like steatohepatitis. Taken together, these findings suggest that an additional metabolic "hit"

like hypercholesterolemia may contribute, in part, to increase the severity of fatty liver disease in populations consuming alcohol and exposed to environmental toxicants like cigarette smoke.

Increased expression of iNOS and production of RNS are associated with inflammation observed in alcohol-induced fatty liver disease [37,51]. NO and its metabolites have been linked to mechanisms of hepatotoxicity through mtDNA damage, as well as through post-translational modification of cellular and mitochondrial proteins via oxidation, nitrosation, and nitration [64-66]. Specifically, excess NO reacts with superoxide ($O_2^{\bullet-}$) to produce $ONOO^-$, a highly reactive species, which has been implicated as a key mediator in NO-dependent cellular and mitochondrial dysfunction via protein nitration [67]. Whereas the levels of iNOS and CYP2E1, a source of $O_2^{\bullet-}$, were elevated over control values by ethanol consumption alone, greater increases were observed in the ethanol + ETS group. Similarly, nitration and mtDNA damage were increased in the ethanol + ETS group compared to other groups. These results are in agreement with those observed in heart tissue of apoE$^{-/-}$ mice exposed to ethanol + ETS, which showed increased iNOS, oxidant damage, and atherosclerosis [26]. Taken together, this suggests a common mechanism involving oxidative and/or nitrative stress in the pathobiology of atherosclerosis and fatty liver disease; two conditions of the cardiometabolic syndrome.

Hypoxia also has been shown to contribute to hepatotoxicity from a number of toxicants including alcohol and with other pathologies linked to steatosis. Studies show that alcohol consumption increases hypoxia in the pericentral region of the liver (i.e. zone 3) due to increased O_2 consumption in response to ethanol oxidation [49]. Similarly, chronic hypoxia associated with obstructive sleep apnea has been implicated as a contributing factor to the development of fatty liver disease [68] and atherosclerosis [69] in obese individuals. It is proposed that the interaction of hypoxia with other stressors or "hits", either metabolic or environmental, may participate in the progression from simple steatosis to steatohepatitis. What hasn't been studied to date is whether combined exposure to ETS exacerbates ethanol-induced hypoxia.

It is well established that a critical adaptation to reduced oxygen availability is upregulation of the transcription factor HIF1α and adaptive pathways to prevent hypoxic injury. Consistent with the histopathology presented herein, is the observation that liver from the ethanol + ETS group showed the highest level of HIF1α expression indicating enhanced hypoxia from this combined toxicant exposure. ETS may enhance liver hypoxia due to the presence of low amounts of carbon monoxide in tobacco smoke, which could have the effect of reducing the amount of oxygen delivery to hepatocytes through formation of carboxyhemoglobin [70]. Similarly, the presence of these multiple "hits" could increase oxygen demand in liver due to increased metabolic activity thereby enhancing tissue hypoxia. This may be particularly relevant given that increased mtDNA damage and cytochrome c oxidase defects were also observed in the ethanol + ETS group. It is possible that one precarious consequence of these damaged mitochondria may be a disturbance in the normal O_2 gradient within the liver lobule resulting in increased hypoxia. In particular, it is proposed that defective mitochondria in the ethanol + ETS group will have increased O_2 uptake to attempt to maintain sufficient ATP levels in hepatocytes for use in detoxification and/or repair pathways. This would result in a "steepening" of the liver O_2 gradient due to increased mitochondrial O_2 consumption in the upstream O_2-rich periportal hepatocytes, which would place the downstream O_2-poor pericentral hepatocytes under increased hypoxia.

Interestingly, these results contribute to an ongoing debate in the literature focused on whether or not small decreases, e.g. 15-30% in the levels or activities of key respiratory chain enzymes like cytochrome c oxidase negatively impact energy conservation and contribute to tissue pathology. While it is evident that impaired ATP synthesis can not be linked to a specific defect

in cytochrome *c* oxidase *per se*, it is possible that a defect in oxidase or one of the other respiratory complexes could alter the respiratory threshold or reserve capacity needed to maintain bioenergetic homeostasis. It is expected that under conditions of pathological stress (i.e. chronic ethanol, ETS, and dyslipidemia) the bioenergetic demands on hepatocytes are likely increased due to increased repair and detoxification while the threshold capacity may be limited due to a loss in cytochrome *c* oxidase function. In support of this hypothesis we and others have shown that similar decreases in state 3 respiration following chronic alcohol consumption is associated with the inability of the mitochondrion to meet the energetic demands of the tissue. Specifically, decreased activity in respiratory chain complexes results in depressed ATP synthesis in liver [71]. Importantly, this can be related directly to cell viability because the inability to maintain adequate ATP levels increases hepatocyte death when steatotic hepatocytes are placed under low oxygen tensions [72]. Whether this occurs in this "multi-hit" model of alcoholic steatosis is not known; however, it is possible because in the current study we observed amplified hepatic hypoxia *in vivo* in the ethanol + ETS group. Therefore, when ATP synthesis is impaired this could enhance hepatocyte death particularly if ATP needs are increased acutely. Moreover, this may be critical for pathology as a loss in the capacity for maintenance of hepatic ATP levels may lead to more serious or permanent damage due to a depression in anabolic processes responsible for replacing damaged and/or lost cellular components.

The interaction of NO with the mitochondrion has been implicated as a key mediator in how cells respond to hypoxic stress. NO modulates mitochondrial function through control of mitochondrial biogenesis [73] and respiration by binding to the heme center in cytochrome *c* oxidase [74,75]. While the interaction of NO and cytochrome *c* oxidase has been proposed to extend O_2 gradients and increase O_2 tensions in healthy tissue via inhibition of respiration [76], recent studies show that under pathologic conditions the inability to adapt through use of this signaling pathway occurs when mitochondria are damaged in cardiac hypertrophy, alcoholic fatty liver disease, and high fat diet hepatotoxicity resulting in ROS/RNS production and bioenergetic defects [77-80]. Previously, we reported that mitochondrial respiration becomes more sensitive to inhibition by exogenous NO exposure in response to chronic alcohol exposure [79] and that this response is iNOS dependent because increased sensitivity is not observed in mitochondria isolated from livers of iNOS$^{-/-}$ mice fed ethanol [37]. Important to this, is the observation that chronic alcohol-mediated steatosis is attenuated in iNOS$^{-/-}$ mice fed ethanol suggesting a link between NO, mitochondria, and pathology [37,51]. Therefore, via iNOS induction and continual NO exposure, chronic alcohol consumption may change how endogenous NO controls mitochondrial respiration leading to enhanced hypoxia, increased ROS/RNS production, and compromised bioenergetics. Similarly, ETS may further exacerbate this alcohol dependent change in NO-control of respiration limiting the capacity of mitochondria to respond to cellular stress. Even though the interactive mechanisms responsible for the effects of hypercholesterolemia, ETS, and/or ethanol on NO biology and adaptation to hypoxia are poorly understood, we propose that toxicant exposure on a dyslipidemic background increases mitochondrial damage and susceptibility to hypoxic injury, which may be a critical and currently unrecognized factor or "hit" in the development of serious liver disease like steatohepatitis.

Summary/conclusions

The findings reported in this study provide strong support for the emerging paradigm that environmental and metabolic stressors, also referred to as "hits", influence the progression from simple fatty liver (i.e. steatosis) to more serious liver diseases observed in the chronic alcohol consumer [6]. This study indicates that pro-inflammatory and pro-oxidant conditions induced by these multiple stressors or "hits" may disrupt the physiologic role of NO signaling and in doing so may contribute, in part, to mitochondrial damage and hypoxic liver injury.

Furthermore, we postulate that it is under these conditions that increased post-translational modification of proteins, particularly mitochondrial proteins, will occur rendering proteins of key metabolic pathways catalytically inactive amplifying bioenergetic defects and increasing liver toxicity. Because the chronic alcohol consumer is likely to suffer from combinations of metabolic disorders and be exposed to multiple environmental toxicants, it will be critical for future investigations to identify the molecular targets and mechanisms through which these potential stressors or "hits" interact to accelerate and worsen alcoholic liver disease.

Acknowledgments

List of abbreviations

ATP	adenosine triphosphate
BN-PAGE	blue native gel electrophoresis
CYP2E1	cytochrome P450 2E1
ETS	environmental tobacco smoke
FA	filtered air
HIF1α	hypoxia inducible factor 1α
iNOS	inducible nitric oxide synthase
LDL	low density lipoprotein
mtDNA	mitochondrial DNA
NO	nitric oxide
3-NT	3-nitrotyrosine
O$_2$	oxygen
ONOO$^-$	peroxynitrite
QPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RNS	reactive nitrogen species
TBS-T	Tris-buffered saline with Tween-20
VLDL	very low density lipoprotein

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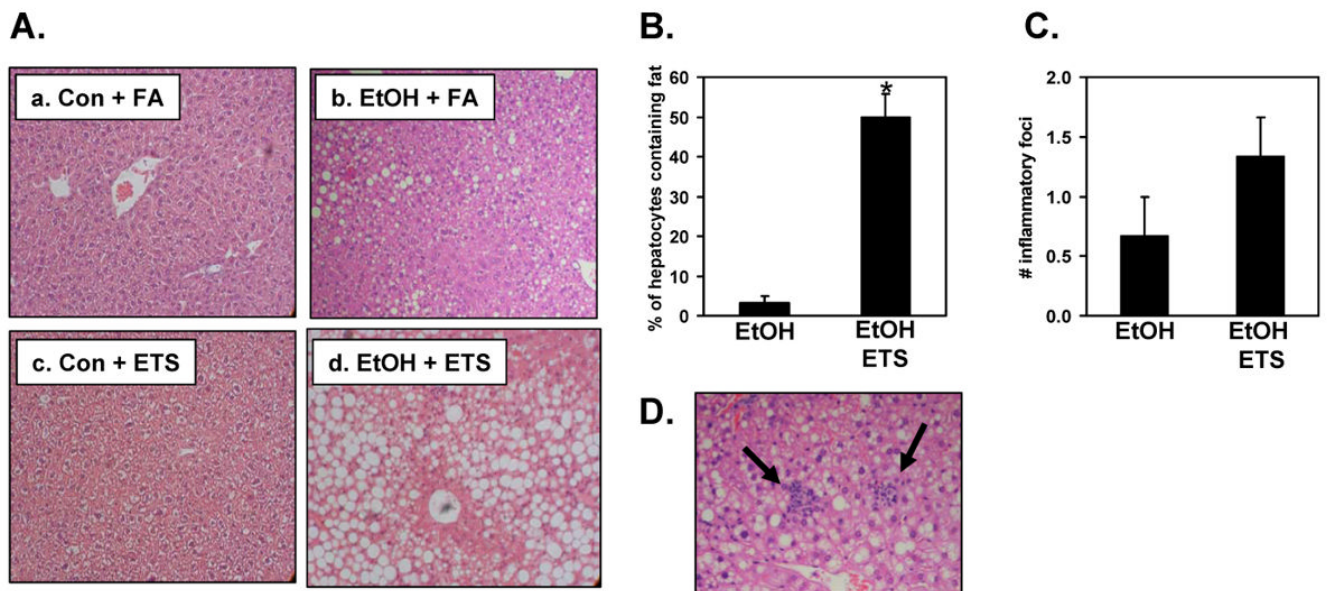
Fig. 1. ETS increases ethanol dependent steatosis and inflammation
Liver was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Panel A, Livers from mice fed control diets (Con) exposed to filtered air (FA) (a) or ETS (c) show no pathological changes or fat deposition in hepatocytes. Mice fed an ethanol-containing diet (EtOH) and exposed to filtered air (b) show mild steatosis, whereas ethanol-fed mice exposed to ETS (d) have severe steatosis. Magnification is 100X. Panel B, Quantification of steatosis. Panel C, Quantification of inflammatory foci. Panel D, Photomicrograph showing aggregates of neutrophils and lymphocytes within liver parenchyma of ethanol + ETS group (arrows). Magnification is 200X.
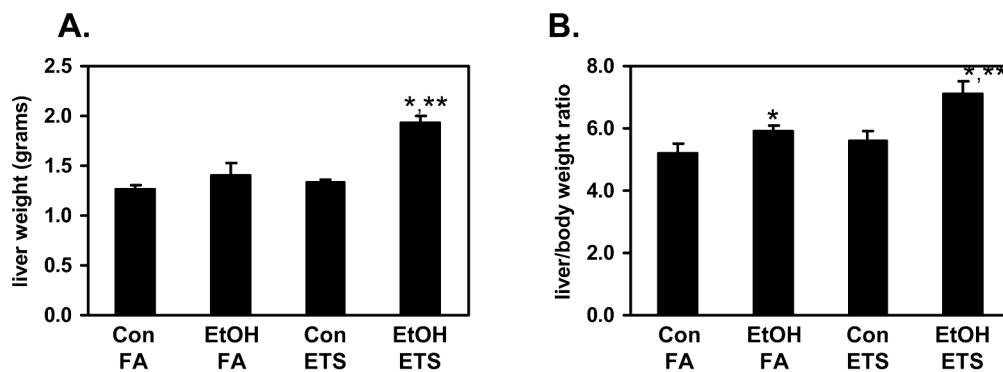
Fig. 2. ETS significantly enhances the ethanol dependent increase in liver weight and liver/bodyweight ratio
Mice were pair-fed control (Con) and ethanol (EtOH)-containing diets for 6 weeks while being exposed to filtered air (FA) or ETS (10 mg TSP/m^3) for the last 4 weeks of the study. ETS exposure significantly increased the liver weight and liver/body weight ratio in ethanol-fed mice. Data represent the mean ± SEM for 6 mice per group. [*]p<0.05, compared to corresponding control; [**]p<0.05, compared to ethanol + filtered air. For liver weight; two-factor ANOVA, ethanol, p=0.0008; ETS, p=0.0023; ethanol × ETS, p=0.017. For liver/body weight ratio; two-factor ANOVA, ethanol, p=0.016; ETS, p=0.042; ethanol × ETS, 0.27.
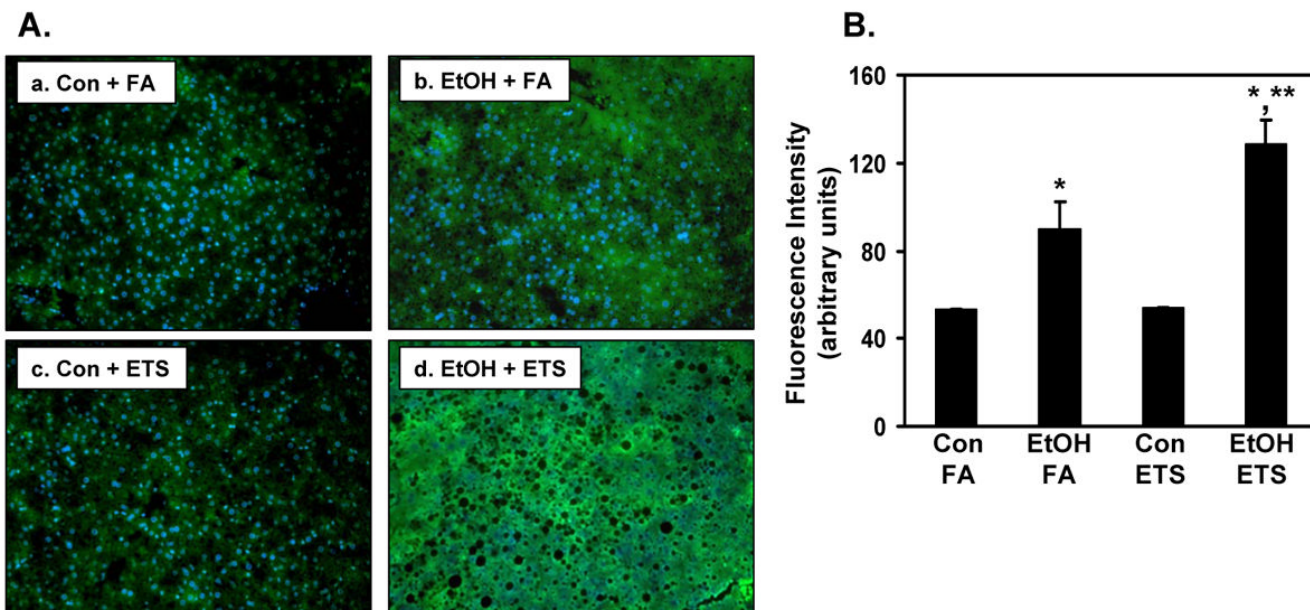
Fig. 3. ETS enhances ethanol-dependent hypoxia in liver
Immunofluorescence was performed on formalin fixed liver tissue using HIF1α antiserum
(green) and nuclei were counterstained with DAPI (blue). No statistical difference in HIF1α
levels was observed between control groups (Con + FA, panel a and Con + ETS, panel c),
whereas there was a significant increase in HIF1α staining in the ethanol + filtered air group
(EtOH + FA, panel b) compared to corresponding control. This increase in HIF1α was
amplified in the ethanol + ETS group (panel d) with widespread HIF1α expression in liver.
Panel B shows quantification with *p<0.05, compared to corresponding controls, and
**p<0.05, compared to ethanol + filtered air. Two-factor ANOVA, ethanol, p=0.00013; ETS,
p=0.043; ethanol × ETS, p=0.048. Data represent the mean ± SEM for n=3 pairs per group.
Magnification is 100X.
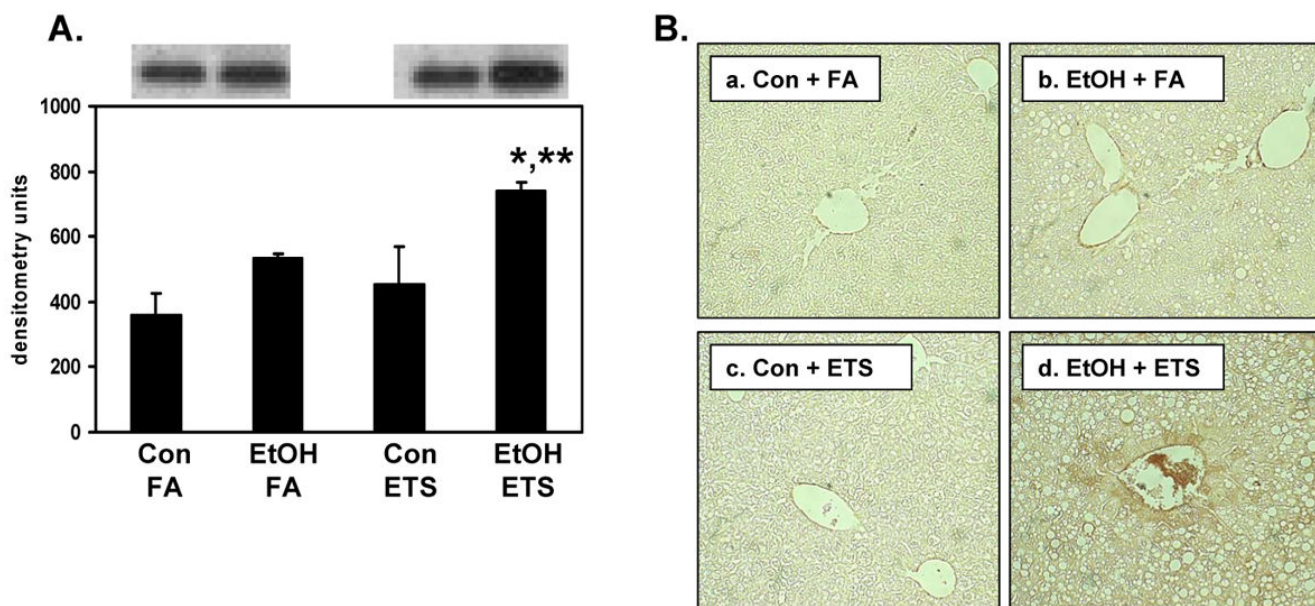
Fig. 4. Induction of alpha smooth muscle actin by ethanol and ETS in liver
Panel A, Liver homogenates were subjected to SDS-PAGE and immunoblotting for quantification of alpha smooth muscle actin protein. The top panel in 4A shows representative immunoblots from one pair of control (Con) and ethanol (EtOH)-fed mice exposed to either filtered air (FA) or ETS. The bottom panel in 4A represents the quantification of alpha smooth muscle actin protein in immunoblots. Panel B, Immunohistochemistry was also performed on formalin fixed tissue and representative photomicrographs from each treatment group are shown: a, Con + FA; b, EtOH + FA; c, Con + ETS; and d, EtOH + ETS. Increased brown staining for alpha smooth muscle actin was observed in the EtOH + ETS group (d) compared to other treatment groups. Data represent the mean ± SEM for n=3 pairs per group. Immunoblot statistics - *p<0.05, compared to corresponding control; **p<0.05, compared to ethanol + filtered air. Two-factor ANOVA; ethanol, p=0.011; ETS, p=0.067; ethanol × ETS, p=0.46. Immunohistochemistry data and statistics – Con + FA, 5.4 ± 0.15; EtOH + FA, 11.4 ± 0.9; Con + ETS, 5.7 ± 0.4; and EtOH + ETS, 16.0 ± 1.9 arbitrary intensity units. Two factor ANOVA; ethanol, p<0.001; ETS, p=0.049; ethanol × ETS, p=0.08. Magnification is 200X.
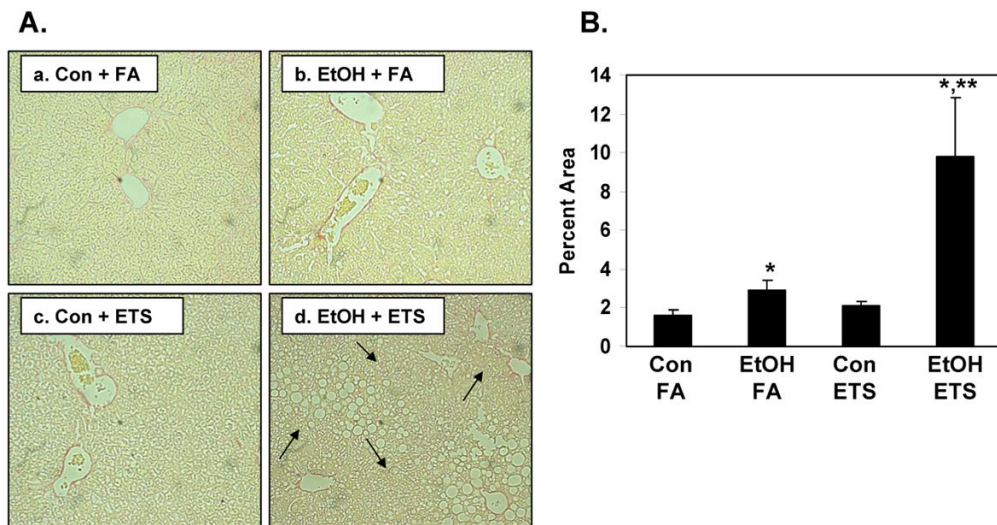
Fig. 5. Increased collagen fibers by ethanol and ETS in liver
Liver sections were stained with Sirius red and analyzed to detect and quantify collagen. Panel A, There was increased Sirius red staining in EtOH + FA group (b) compared to control diet alone (a). Increased Sirius red staining was observed in the EtOH + ETS group (d) with increased collagen detected around the central veins that radiated out into the parenchyma (arrows, panel d). Panel B, Image analysis (percent area) demonstrated increased Sirius red staining in liver from EtOH + ETS group compared to all other groups. Data represent the mean ± SEM for 3 pairs of mice. Magnification is 200X. *p<0.05, compared to each corresponding control; **p<0.05, compared to ethanol + filtered air. Two-factor ANOVA; ethanol, p=0.019; ETS, p=0.044; ethanol × ETS, 0.074.
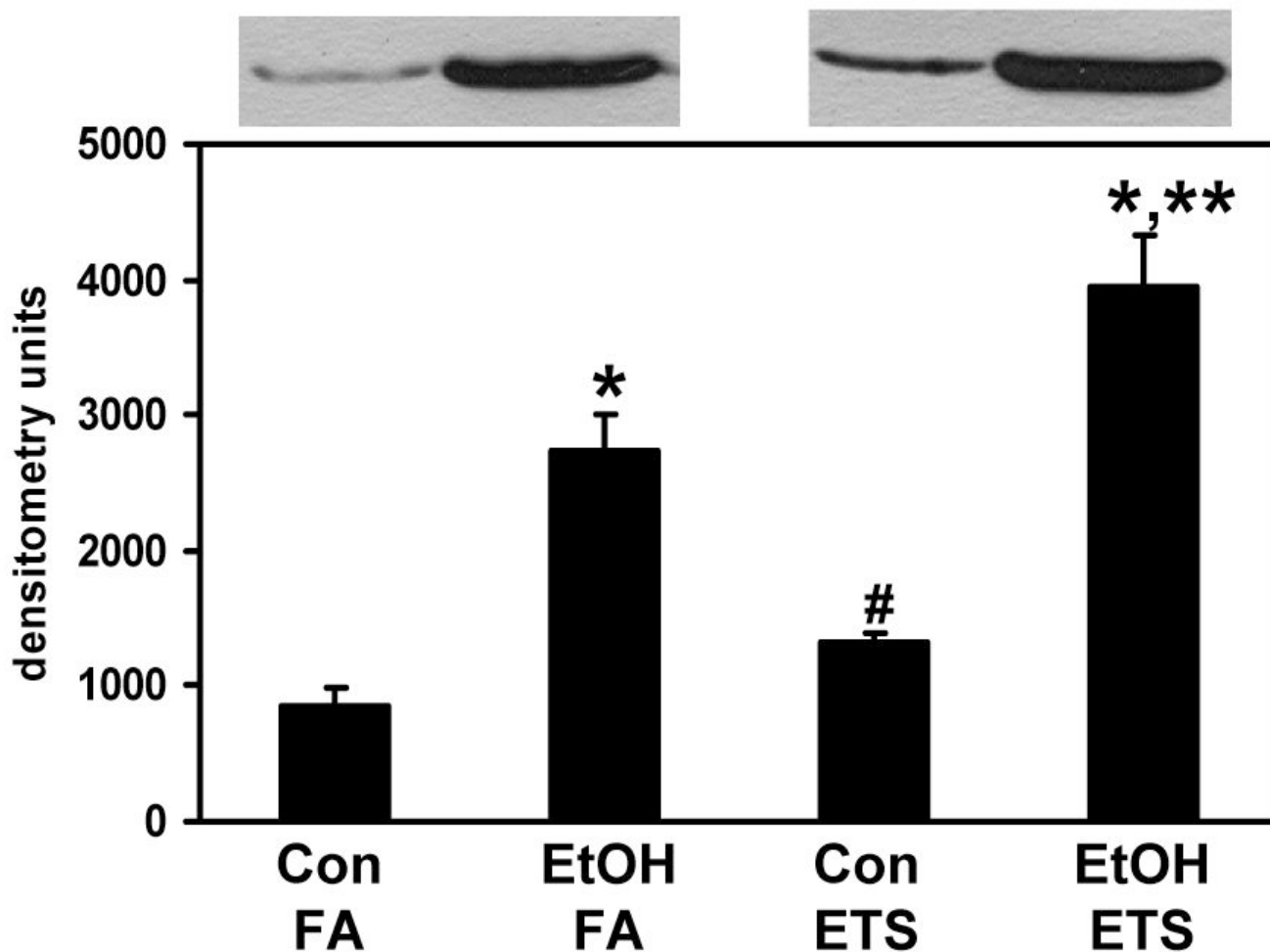
Fig. 6. Combined exposure to ethanol and ETS significantly increases hepatic levels of CYP2E1 protein
Liver homogenates were subjected to SDS-PAGE and immunoblotting for quantification of CYP2E1 protein. Top panel shows representative immunoblots from one pair of control (Con) and ethanol (EtOH)-fed mice exposed to either filtered air (FA) or ETS. Bottom panel represents the quantification of CYP2E1 protein. Data represent the mean $\pm$ SEM for n=3 pairs per group. *$p<0.05$, compared to corresponding control; **$p<0.05$, compared to ethanol + filtered air; #$p<0.05$, compared to control + filtered air. Two-factor ANOVA; ethanol, $p<0.0001$; ETS, $p=0.008$; ethanol $\times$ ETS, 0.17.
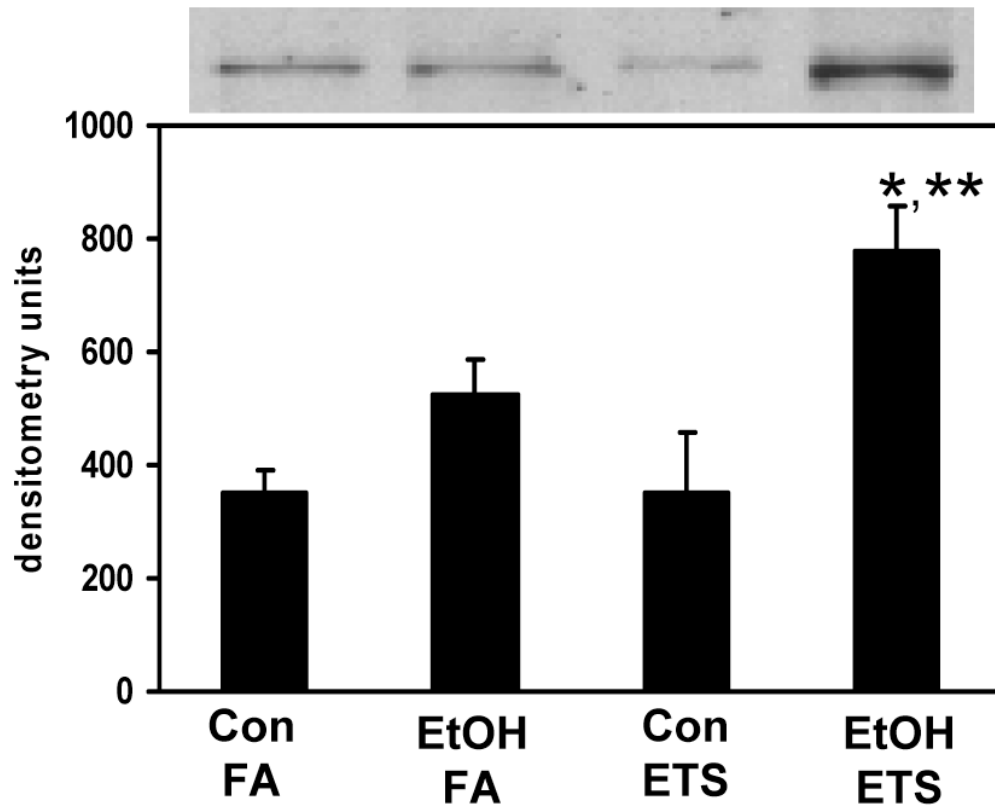
Fig. 7. Exposure to ethanol and ETS significantly increases hepatic levels of iNOS
Liver homogenates were subjected to SDS-PAGE and immunoblotting for quantification of iNOS protein. Top panel shows representative immunoblots from one pair of control (Con) and ethanol (EtOH)-fed mice exposed to either filtered air (FA) or ETS. Bottom panel represents the quantification of iNOS. Data represent the mean ± SEM for n=3 pairs per group. *p<0.05, compared to corresponding control; **p<0.05, compared to ethanol + filtered air. Two-factor ANOVA; ethanol, p=0.002; ETS, p=0.13; ethanol × ETS, p=0.12.
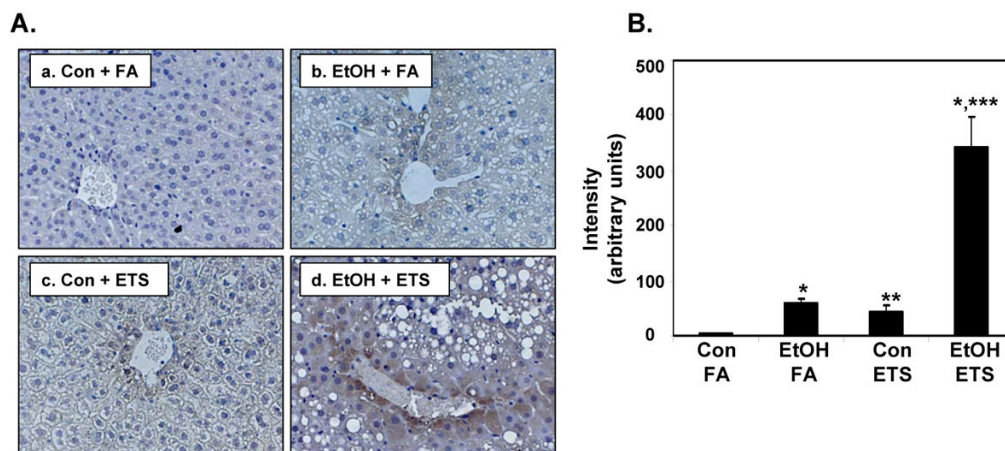
Fig. 8. Hepatic levels of 3-NT are increased by exposure to ethanol and ETS
Immunohistochemistry was performed on formalin fixed tissues using 3-NT antiserum (brown) against a hemtoxylin nuclear counterstain (blue) in liver sections of control (Con) and ethanol (EtOH)-fed mice exposed to either filtered air (FA) or ETS. Panel A, There was increased 3-NT in control + ETS group (c) and the EtOH + FA group (b) compared to control diet alone (a). Increased 3-NT was observed in the EtOH + ETS group (d) with intense staining for 3-NT around the central veins. Panel B, Image analysis demonstrated increased intensity of 3-NT in liver from EtOH + ETS group compared to all other groups. Data represent the mean ± SEM for 3 pairs of mice. Magnification is 200X. *p<0.05, compared to each corresponding control; **p<0.05, compared to control + filtered air; ***p<0.05, compared to ethanol + filtered air. Two-factor ANOVA; ethanol, ETS, and ethanol × ETS, p<0.0001, ethanol, ETS.
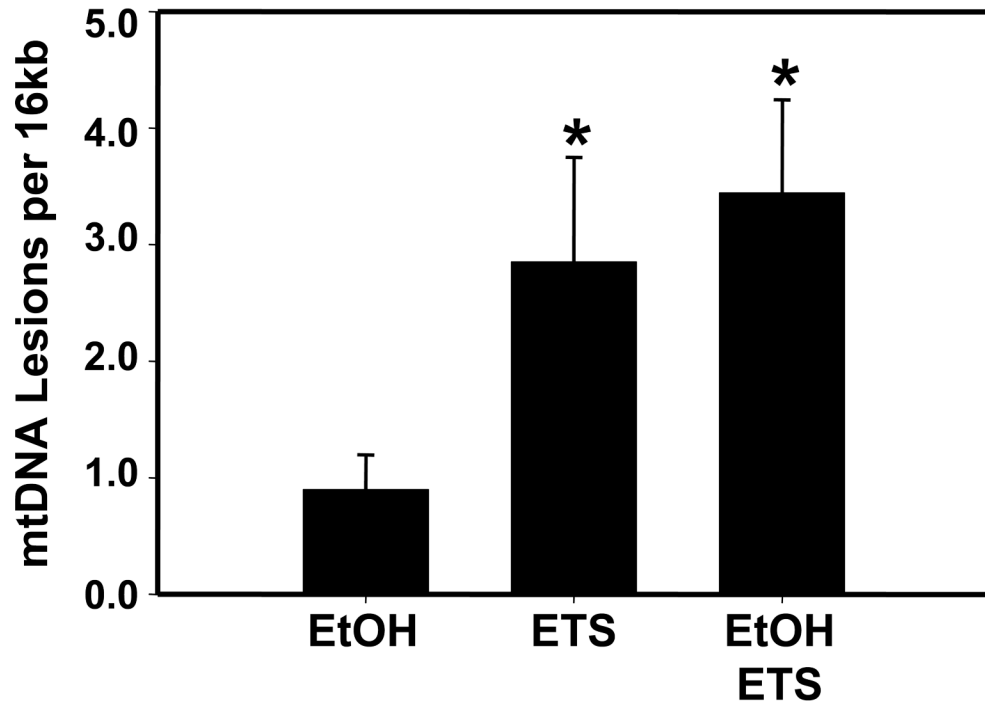
Fig. 9. Liver mitochondrial DNA damage is increased by ethanol and ETS
Genomic DNA was prepared from livers and mtDNA damage was assessed using QPCR. Data presented in the bar graph indicate the relative amount of mtDNA damage (lesions per 16 kb) in liver from ethanol-fed (EtOH), ETS exposed, and EtOH + ETS animals compared with their corresponding controls. Controls are set at zero lesions per 16kb. Data represent the mean ± SEM for n=3 pairs animals per group, *p<0.05, compared to corresponding control.
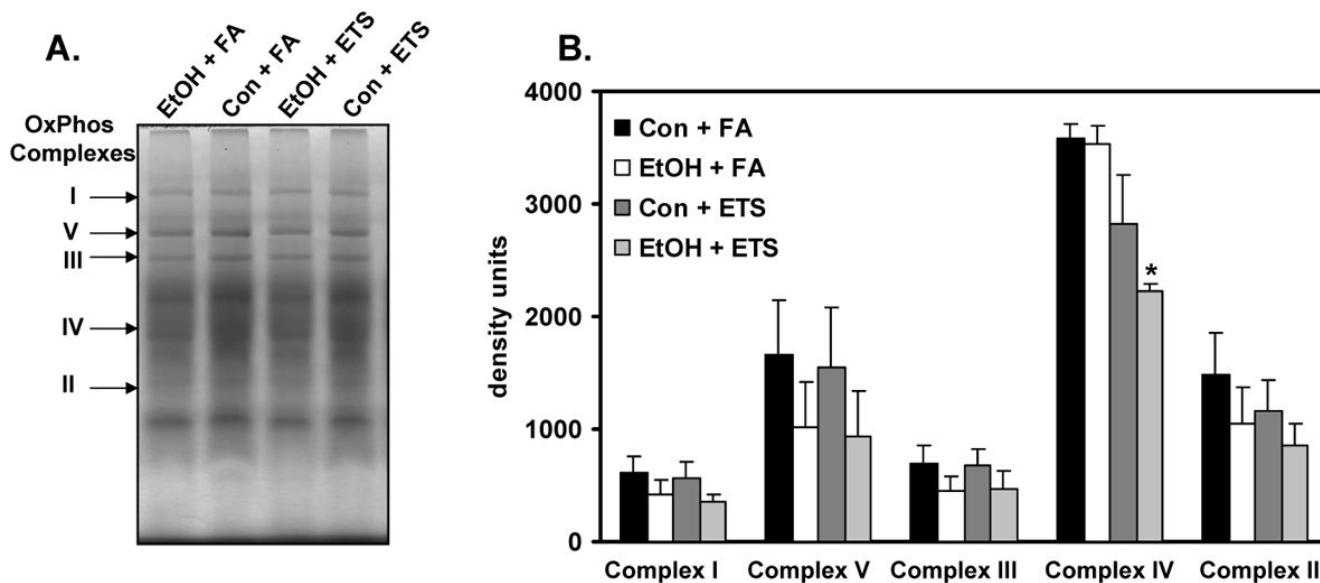
Fig. 10. Ethanol and ETS decrease cytochrome *c* oxidase in liver
Representative BN-PAGE gels of mitochondria from control + filtered (Con + FA), ethanol +
filtered air (EtOH + FA), control + ETS (Con + ETS), and ethanol + ETS (EtOH + ETS) are
shown in panel A. For these gels, 250 µg of mitochondrial protein was loaded onto non-
denaturing 5-15% gradient gels to separate the oxidative phosphorylation complexes intact and
in native form. The complexes are labeled by the arrows. Panel B presents a comparison of the
relative densities (i.e. quantities) of complexes I, V, III, IV, and II from each of the 4 treatment
groups. Data represent the mean ± SEM for 3 pairs per group. *p=0.022, compared to ethanol
+ filtered air group. Two-factor ANOVA, ethanol, p=0.55; ETS, p=0.015; ethanol × ETS,
p=0.66.