

Potential Contributions of the Tobacco Nicotine-Derived Nitrosamine Ketone (NNK) in the Pathogenesis of Steatohepatitis in a Chronic Plus Binge Rat Model of Alcoholic Liver Disease

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Abstract — **Aims:** Alcoholic liver disease (ALD) is linked to binge drinking and cigarette smoking. Heavy chronic ± binge alcohol, or low-level exposures to dietary nitrosamines cause steatohepatitis with insulin resistance and oxidative stress in animal models. This study examines hepatotoxic effects of sub-mutagenic exposures to tobacco-specific nitrosamine (NNK) in relation to ALD. **Methods:** Long Evans rats were fed liquid diets containing 0 or 26% (caloric) ethanol (EtOH) for 8 weeks. In Weeks 3 through 8, rats were treated with NNK (2 mg/kg) or saline by i.p. injection, 3×/week, and in Weeks 7 and 8, EtOH-fed rats were binge-administered 2 g/kg EtOH 3×/week; controls were given saline. **Results:** EtOH ± NNK caused steatohepatitis with necrosis, disruption of the hepatic cord architecture, ballooning degeneration, early fibrosis, mitochondrial cytopathy and ER disruption. Severity of lesions was highest in the EtOH+NNK group. EtOH and NNK inhibited insulin/IGF signaling through Akt and activated pro-inflammatory cytokines, while EtOH promoted lipid peroxidation, and NNK increased apoptosis. *O*⁶-methyl-Guanine adducts were only detected in NNK-exposed livers. **Conclusion:** Both alcohol and NNK exposures contribute to ALD pathogenesis, including insulin/IGF resistance and inflammation. The differential effects of EtOH and NNK on adduct formation are critical to ALD progression among alcoholics who smoke.

INTRODUCTION

Alcoholism is a costly public health problem that continues to grow, especially among women and young people (Paula *et al.*, 2010). On a global scale, alcohol abuse is a leading cause of liver-associated morbidity and mortality (Paula *et al.*, 2010; McCullough *et al.*, 2011; Miller *et al.*, 2011). Acute alcohol-related liver injury is usually reversible (Seth *et al.*, 2011), but in some individuals who engage in heavy drinking over long periods of time, steatohepatitis becomes chronic and progressive, ultimately leading to cirrhosis, liver failure, or hepatocellular carcinoma (O'Shea *et al.*, 2010). Mechanistically, chronic alcoholic liver disease (ALD) is linked to injury mediated by complex inter-related pathophysiological processes including: insulin resistance (de la Monte *et al.*, 2008; Pang *et al.*, 2009; Longato *et al.*, 2012), cytotoxicity and lipotoxicity (McVicker *et al.*, 2006; de la Monte *et al.*, 2009a; Cohen and Nagy, 2011; Derdak *et al.*, 2011), inflammation (Ronis *et al.*, 2008; Cohen and Nagy, 2011), oxidative and endoplasmic reticulum (ER) stress (Kaplowitz and Ji, 2006; Pandol *et al.*, 2010; Feldstein and Bailey, 2011; Malhi and Kaufman, 2011; Ramirez *et al.*, 2013), metabolic and mitochondrial dysfunction (Purohit *et al.*, 2009; Ding *et al.*, 2011), decreased DNA synthesis (Sasaki *et al.*, 1994; Banerjee *et al.*, 1998; Pang *et al.*, 2009), and increased cell death (Derdak *et al.*, 2011) (see Supplementary Table S1 for list of abbreviations). ALD progression is marked by activation of pro-fibrogenic pathways (Cohen and Nagy, 2011), setting the stage for cirrhosis and finally liver failure (Vidali *et al.*, 2008; Purohit *et al.*, 2009). Improved understanding of factors contributing to the switch from acute to chronic progressive ALD is needed to identify individuals at risk and refine diagnostic and therapeutic approaches.

Emerging evidence suggests roles for two additional factors in the pathogenesis of ALD: binge drinking and tobacco abuse.

First, acute alcoholic hepatitis typically develops in chronic heavy alcohol drinkers who also binge (Seth *et al.*, 2011). Recent publications demonstrated that better models of ALD could be generated in rodents by binge administering alcohol to chronic ethanol-fed mice (Bertola *et al.*, 2013a,b). In addition, a very high percentage of heavy drinkers (up to 80%) also abuse tobacco products, typically by smoking cigarettes (Romberger and Grant, 2004). Moreover, even among non-smokers, second-hand smoke poses significant health risks due to inadvertent exposures to tobacco nitrosamines and their metabolites (Upadhyaya *et al.*, 2009; Thomas *et al.*, 2011). The overwhelming interest in studying adverse effects of alcohol-tobacco dual exposures has focused on carcinogenesis (Johnson *et al.*, 1996; de Boer *et al.*, 1997; Tramacere *et al.*, 2010; Duell, 2012), including hepatocarcinogenesis (Kuper *et al.*, 2000; Seitz and Stickel, 2006; Ha *et al.*, 2012; Purohit *et al.*, 2013), and the roles of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolites (Go *et al.*, 2005; Duell, 2012).

The concept that NNK and its metabolites could also contribute to ALD pathogenesis and progression stemmed from previous work showing that limited sub-mutagenic doses of other nitrosamines, i.e. streptozotocin (Bolzan and Bianchi, 2002; Koulmanda *et al.*, 2003; Wang *et al.*, 2011) or N-nitrosodiethylamine (NDEA) (de la Monte *et al.*, 2009b; Tong *et al.*, 2009), cause steatohepatitis with hepatic insulin resistance, increased DNA damage, lipid peroxidation, mitochondrial dysfunction, ER stress, and impaired signaling through PI3K-Akt (de la Monte *et al.*, 2009b; Tong *et al.*, 2009). Moreover, although tobacco smoke contains other toxins/carcinogens, e.g. acetaldehyde and acrolein, which generate covalent adducts (Salaspuro, 2011), there are no published reports demonstrating their roles in the pathogenesis of hepatic insulin resistance. The present study tests the hypothesis

that chronic exposure to NNK causes steatohepatitis and exacerbates the effects of chronic+binge ethanol exposures on the histological, biochemical and molecular features of experimental ALD.

METHODS

Experimental model

Long Evans male rats were pair-fed for 8 weeks with isocaloric liquid diets containing 0 or 6% (v/v) ethanol. From Weeks 3 through 8, rats in each group were administered intraperitoneal (i.p.) injections (100 μ l) of NNK (2 mg/kg) or saline on Mondays, Wednesdays and Fridays, and in Weeks 7 and 8, ethanol-fed rats were binged with 2 g/kg ethanol (100 μ l, i.p.) on Tuesdays, Thursdays and Saturdays while controls were treated with saline. At the end of the experiment, blood alcohol levels (tail vein) were measured at 7 AM (basal) and 30 min after binging. Serum was obtained to measure electrolytes, lipids, liver function enzymes, and standard proteins (Marshfield Labs, Marshfield, WI, USA), or endotoxin immunoreactivity (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, San Jose, CA, USA).

Liver collection, processing and storage

Portions of fresh liver were fixed in formalin or Karnovsky's, or snap frozen and stored at -80°C . Fixed tissue was divided for paraffin embedding and cryostat sectioning. Adjacent paraffin sections were stained with Hematoxylin and Eosin (H&E), Sirius Red (fibrosis) (Baumgardner *et al.*, 2008). Cryostat sections were stained with Oil-Red O (ORO; lipids) to visualize hepatic steatosis (Caldwell *et al.*, 2010). Karnovsky's fixed tissue was post-fixed in osmium tetroxide and embedded in Spurr's epoxy resin. Ultra-thin (50–60 nm) sections were stained and processed for electron microscopy.

Hepatic lipid and collagen assays

Cholesterol and triglycerides were measured in liver lipid extracts (Setshedi *et al.*, 2011) using the Analox Instruments GM7 Analyzer. Hepatic collagen was measured using the Sirius Red/Fast Green Collagen Staining Kit (Chondrex, Inc., Richmond, VA, USA).

Enzyme-linked immunosorbent assays

Competitive enzyme-linked immunosorbent assays (ELISAs) measured hepatic Keratin-18 (USCN Life Science, Inc., Hubei, PRC), protein carbonyl, 4-hydroxy-2-nonenal (HNE), and isoprostanol 8-iso-PGF 2α (Cell Biolabs, Inc., San Diego, CA, USA). Luminex bead-based multiplex ELISAs proteins and phosphoproteins through insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, ribosomal protein S6 kinase (p70S6K) and glycogen synthase kinase 3 β (GSK-3 β). Hepatic cytokines were measured using a Rat Cytokine 23-Plex bead-based immunoassay (Bio-Rad, Hercules, CA, USA).

O⁶-methyl-Guanine detection

DNA isolated from rat lung and liver (Lao *et al.*, 2006; Zhang *et al.*, 2009) was subjected to acid hydrolysis (Upadhyaya *et al.*, 2009) and quantified by high-performance liquid chromatography (HPLC). O⁶-methyl-Gua was detected and quantified

by liquid chromatography (LC)-tandem Mass Spectrometry (MS/MS) by capillary LC/electron spray (ESI)-MS/MS using a triple quadrupole mass spectrometer. The ESI source was operated in the positive ion mode. Adducts were quantified with SRM at m/z 166.09 \rightarrow 149.09 for O⁶-methyl-Gua and at the corresponding transition m/z 169.11 \rightarrow 152.12 for [CD $_3$] O⁶-methyl-Gua. DNA levels were calculated from the guanine content determined by HPLC-UV spectrometric analysis as previously described (Lao *et al.*, 2006). (See Supplementary Detailed Methods).

Statistics

All assays were performed with 8 or 10 samples per group. Data depicted in box plots reflect group medians (horizontal bar), 95% confidence interval limits (upper and lower box limits) and range (whiskers). Data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA), and inter-group comparisons were made by repeated measures analysis of variance (ANOVA) with *post hoc* multiple comparison test.

RESULTS

Chronic plus binge ethanol plus tobacco nitrosamine (NNK) exposure model

Over the course of the study, weight gain was similar among the four groups (Supplementary Fig. S1A), and although the final mean body weight of chronic+binge ethanol exposed rats was somewhat reduced relative to the other groups, the differences did not reach statistical significance. Blood alcohol concentrations were measured at 7 AM (basal) and 30 min after the i.p. binge administration of ethanol (2 g/kg) or saline (vehicle). Representative results are shown in Supplementary Fig. S1C and E. As expected, baseline blood alcohol levels were significantly higher in chronic ethanol-fed relative to control rats. However, dual exposures to ethanol and NNK resulted in lower basal blood alcohol levels compared with ethanol feeding alone (Supplementary Fig. S1C). Binge administration of ethanol further increased the blood alcohol concentrations by 2- to 4-fold, but with somewhat lower mean levels in the ethanol+NNK relative to ethanol+vehicle treated rats (Supplementary Fig. S1E). Mean fasting blood glucose levels were higher in all experimental groups relative to control, but the differences only reached significance for rats treated with NNK \pm ethanol (Supplementary Fig. S1F).

Serum chemistry assays were used to measure electrolytes, protein content, and indices of hepatic and renal function (Supplementary Table S2). Results were analyzed by ANOVA with *post hoc* Dunnett (relative to control) and Fisher LSD (multiple comparisons) tests. Most of the differences from control were driven by ethanol exposure, with or without NNK treatments. The only exceptions occurred with respect to ALT and LDH which were significantly reduced by NNK without chronic+binge ethanol feeding. The *post hoc* Fisher test further demonstrated significant differences between NNK and ethanol treatments and ethanol versus ethanol+NNK or NNK versus ethanol+NNK exposures. The most striking effects of NNK versus control were to reduce serum ALT and LDH levels. Ethanol alone increased serum potassium and the anion gap, and reduced serum chloride, bicarbonate, phosphorous,

albumin, creatinine, ALT, Alkaline phosphatase, and LDH. The significant effects of ethanol+NNK relative to control were to increase serum sodium, globulin, urea/creatinine ratio, and anion gap, and reduce chloride, bicarbonate, phosphorous, albumin, albumin/globulin ration, ALT, and alkaline phosphatase. It is noteworthy that the maximum effects of ethanol on ALT and LDH may not have been measured due to the short interval (30 min) in which blood was sampled after binge ethanol administration. The levels of endotoxin immunoreactivity in serum were uniformly low and not significantly different among the groups (data not shown).

Ethanol and NNK-associated liver pathology

Although the mean liver weights were similar among the groups (Supplementary Fig. S1B), the calculated liver/body weight ratios were higher in the ethanol and ethanol+NNK groups (Supplementary Fig. S1D). Macroscopic examination of the fresh livers revealed the expected reddish-brown parenchyma control specimens, and swelling with parenchymal pallor following ethanol, NNK or both exposures (Supplementary Fig. S2). Histological sections stained with H&E revealed the expected

well-delineated chord-like architecture with uniform populations of hepatocytes in control livers (Fig. 1A). NNK and/or ethanol exposures resulted in loss of the regular hepatic chord-like architecture (Fig. 1B–D), foci of necrosis (Fig. 1B1 and C3), lymphomononuclear cell inflammation (Fig. 1B2 and C2), steatosis (Fig. 1B3, C2 and D2), hepatocellular apoptosis (Fig. 1C4 and D3-inset), pyknosis (Fig. 1B4, C4 and D4) and ballooning degeneration (Fig. 1D3) of hepatocytes.

In control livers, ORO staining for lipid was modest and mainly distributed in Zone 3 (Supplementary Fig. S3A). NNK treatment resulted in mild but diffusely increased ORO staining throughout the hepatic lobules (Supplementary Fig. S3B). Chronic+binge ethanol, with or without NNK treatment resulted in strikingly increased pan-lobular ORO staining throughout the liver (Supplementary Fig. S3C and D). Correspondingly, hepatic triglyceride (Fig. 2A) and cholesterol (Fig. 2B) levels were significantly elevated by the ethanol \pm NNK exposures relative to control or NNK-only livers. NNK alone slightly increased hepatic triglyceride and cholesterol content relative to control, but the differences were not statistically significant.

Sirius Red staining was used to detect collagen deposition as an indicator of early fibrosis. Control livers had minimal or no

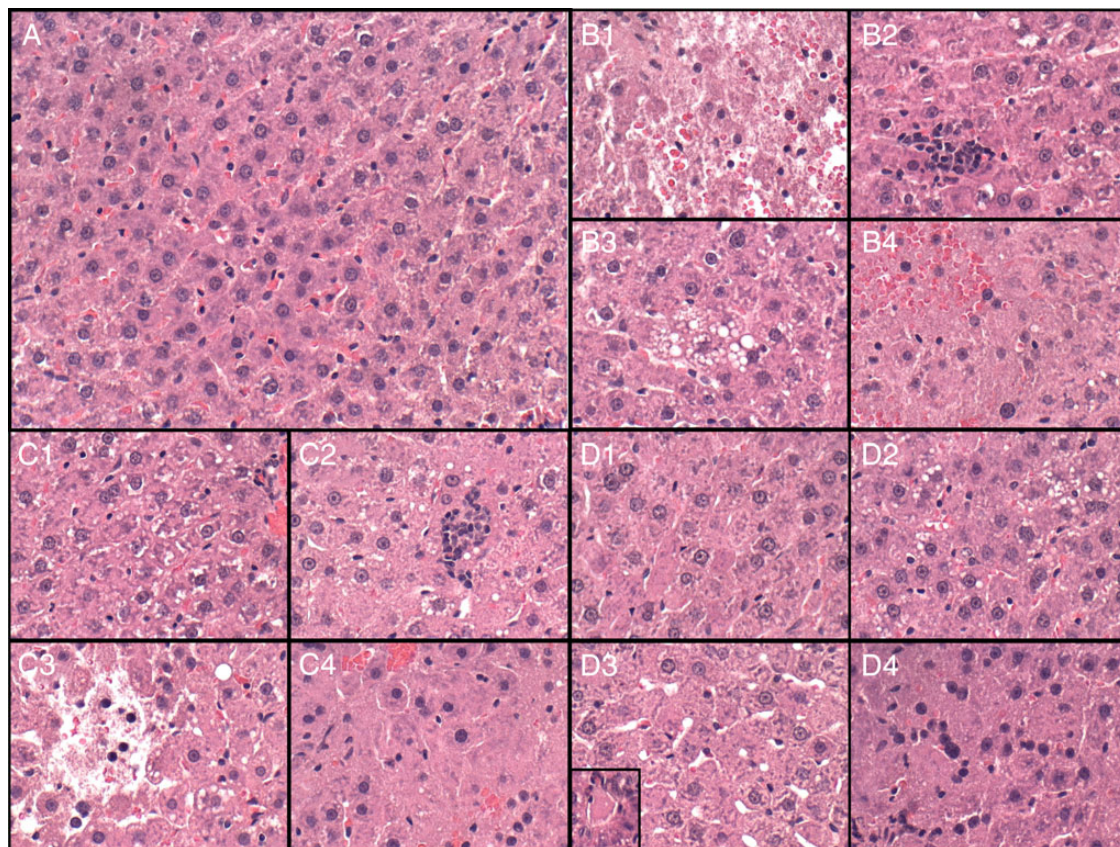


Fig. 1. Ethanol and NNK effects on liver histology. Long Evans rats fed with isocaloric liquid diets containing 0 or 26% ethanol for 9 weeks were treated with NNK (2 mg/kg) or vehicle (3 \times /week) during the last 6 weeks, and binged with ethanol (2 g/kg) or saline (3 \times /week) in the last 2 weeks. Formalin-fixed paraffin-embedded sections of liver were stained with H&E. (A) Control+vehicle liver with normal chord-like architecture, uniform hepatocytes, and no inflammation or necrosis. (B) Control+NNK liver with (B1) necrosis (pale area with shrunken nuclei), (B2) lobular inflammation, (B3) steatosis-vacuoles in center, and (B4) apoptosis with reduced nuclear density, nuclear pleomorphism (pyknotic nuclei, variability in size, homogenization of cytoplasm), and loss of the chord-like architecture. (C) Ethanol diet+vehicle liver with (C1) loss of hepatic chord architecture, nuclear fading or loss, and microvesicular steatosis (vacuoles), (C2) lobular inflammation and microsteatosis (small vacuoles), (C3) focal necrosis, and (C4) marked distortion of hepatic architecture with apoptosis, reduced nuclear density, and loss of the chord-like architecture. (D) Ethanol diet+NNK treated liver with (D1) drop-out of nuclei, (D2) microsteatosis, (D3, D4) apoptosis, marked distortion of hepatic architecture, and ballooning degeneration (D3-inset). Original magnification-all \times 200.

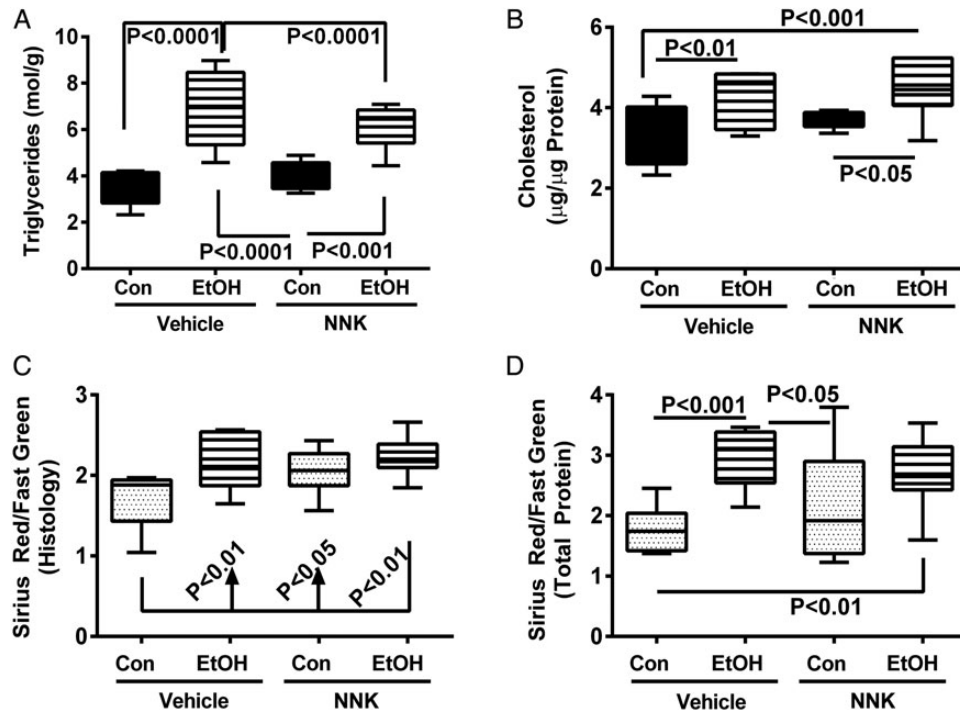


Fig. 2. Quantitative assessments of hepatic lipid and collagen content. (A) Triglycerides and (B) cholesterol were measured in liver lipid extracts using the AnaloX 7 protocol and apparatus with results normalized to sample weight (triglycerides) or protein content (cholesterol). The Sirius Red/Fast Green assay was used to quantify collagen content in (C) histological sections or (D) aqueous tissue homogenates (see Supplementary Methods). Inter-group comparisons were made by two-way ANOVA. Results of *post hoc* Tukey multiple comparison tests are shown in the panels.

detectable inter-hepatocyte or sinusoidal fibrosis (Supplementary Fig. S4A). NNK treatment mildly increased collagen fibril abundance, manifested by short Sirius red-stained strands along sinusoids and between hepatocytes (Supplementary Fig. S4B). More prominent fibrosis was observed with chronic+binge ethanol exposures (Supplementary Fig. S4C), and superimposed treatment with NNK further increased collagen fibril abundance (Supplementary Fig. S4D). Fibrosis was quantified by measuring relative levels of collagen in histological sections (Fig. 2C) and aqueous liver homogenates (Fig. 2D) using a commercial Sirius Red/Fast Green assay. Both methods revealed significant ethanol-associated increases in hepatic collagen (fibrosis). In addition, in the NNK-exposed livers, significantly elevated levels of collagen were detected in histological sections but not in homogenates suggesting that the histological section assay may be more sensitive.

Transmission electron microscopic studies demonstrated that NNK treatment reduced the abundance and altered the distribution of mitochondria, reduced nuclear chromatin, and increased glycogen content and degenerative vacuoles in the cytoplasm relative to control (Supplementary Fig. S3A-a and b). Ethanol had similar effects but also increased cytoplasmic lipid vacuoles (Fig. 3A-c). Combined ethanol+NNK exposures caused striking reductions and alterations in mitochondria and markedly increased intra-hepatocyte lipid droplets (Fig. 3A-d). Higher magnification images demonstrated the expected parallel stacks of rough endoplasmic reticulum (RER) in close juxtaposition with mitochondria in control livers (Fig. 3B-a), and reduced physical associations between mitochondria and RER in the NNK, ethanol and ethanol+NNK groups (Fig. 3B-b–d). Moreover, depletion of RER was most conspicuous in the ethanol+NNK group. In the experimental

groups, loss of RER integrity with gaps, dilatations and disorganization of structure was further illustrated at high magnification (Fig. 3C-a–d). It is noteworthy that we did not detect NNK- or ethanol-associated alterations in mitochondrial structure.

Ethanol and NNK effects on pro-inflammatory mediators

We used a 23-plex ELISA to assess the effects of chronic+binge ethanol, NNK, or both exposures on inflammatory mediators in liver. The results demonstrated broadly increased expression of pro-inflammatory cytokines in livers of all three experimental groups relative to control. The only reductions were noted with respect to G-CSF and RANTES, while VEGF was not significantly affected by the treatments (Supplementary Table S3). NNK, ethanol and NNK+ethanol all significantly increased hepatic levels of GM-CSF, GRO/KC, IFN- γ , IL-10, IL-13, IL-18, IL-1 α , IL-2, IL-4, IL-7, MCP-1 and MIP-3a. Ethanol and Ethanol+NNK increased hepatic expression of EPO, IL-17A, M-CSF and TNF- α . Ethanol exposure alone increased hepatic IL-12 and IL-1 β , while NNK only exposure increased expression of IL-6 and IL-1 β . There were no instances in which the effects of ethanol and NNK appeared to be additive. Instead, the responses were independently driven by ethanol or NNK, but generally more prominently by ethanol, and not further by dual exposures (Supplementary Table S3).

Ethanol and NNK effects indices of cellular injury and O⁶-methyl-Gua adducts

We used competitive ELISAs to measure immunoreactivity to Keratin-18, protein carbonyls, 4-hydroxy-2-nonenal (4-HNE),

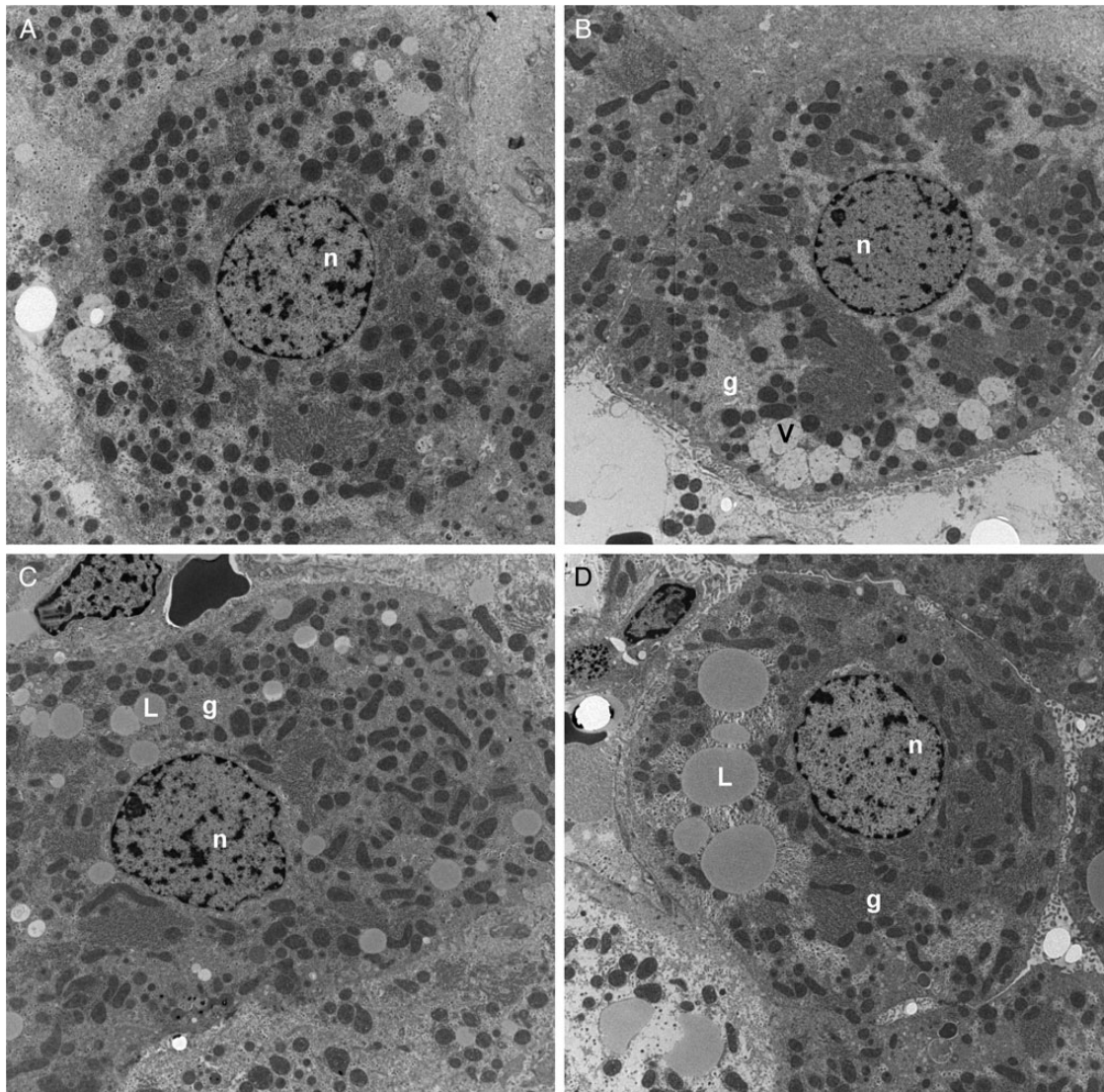


Fig. 3A. Transmission electron microscopic studies. (A) Low magnification images showing reductions in mitochondria and nuclear chromatin (n), and increased lipid droplets (L), degenerative vacuoles (V), and glycogen (g) in (b) NNK-, (c) ethanol, and (d) ethanol+NNK treated livers relative to (a) control (original magnification $\times 3500$).

and 8-epimer of Prostaglandin F 2α (8-iso-PGF 2α) (Fig. 4). Two-way ANOVA tests demonstrated significant ethanol effects for 4-HNE and 8-iso-PGF 2α , NNK effects for all but protein carbonyl and lipid peroxidation adducts, and no significant ethanol \times NNK interactive effects (Supplementary Table S4). *Post hoc* Tukey repeated measures tests demonstrated that Keratin-18 immunoreactivity was increased in both the ethanol+vehicle and ethanol+NNK exposure groups relative to control+vehicle (Fig. 4A), but no significant inter-group differences were obtained with respect to protein carbonyl adduct accumulation (Fig. 4B). Hepatic 8-iso-PGF-2 α (Fig. 4D) adducts were significantly elevated by NNK exposures, independent of ethanol. HNE immunoreactivity was highest in the ethanol+NNK exposed livers (Fig. 4C), the only convincing example of an additive effect of NNK and ethanol. Two samples each of lung and liver from control or ethanol+NNK treated rats were analyzed for *O*⁶-methyl-Gua adducts. None of the control samples had detectable *O*⁶-methyl-Gua, whereas similarly high levels of *O*⁶-methyl-Gua were detected in lung

(2836 and 1147 fmol/mg DNA) and liver (1434 and 1797 fmol/mg DNA) from the ethanol+NNK group.

Ethanol and NNK effects on mediators of insulin and IGF-1 signaling in liver

The levels of total and phosphorylated insulin receptor, IGF-1 receptor, IRS-1, Akt, GSK-3 β , and p70S6K were measured using 7-Plex bead-based assays. Relative phosphorylation of insulin/IGF-1, IRS-1 Akt, GSK-3 β signaling proteins was determined from the calculated ratios of phosphorylated/total proteins. Results of two-way ANOVA tests are shown in Supplementary Table S5, and the corresponding graphs are shown in Figs. 5 and 6 and Supplementary Fig. S5. Ethanol and NNK exposures had significant effects on insulin receptor, IRS-1 and GSK-3 β protein levels, whereas only NNK had significant effects on p70S6K protein expression. Ethanol had significant effects on phosphorylated and relative phosphorylated levels of insulin receptor, Akt, GSK-3 β , and p70S6K, and

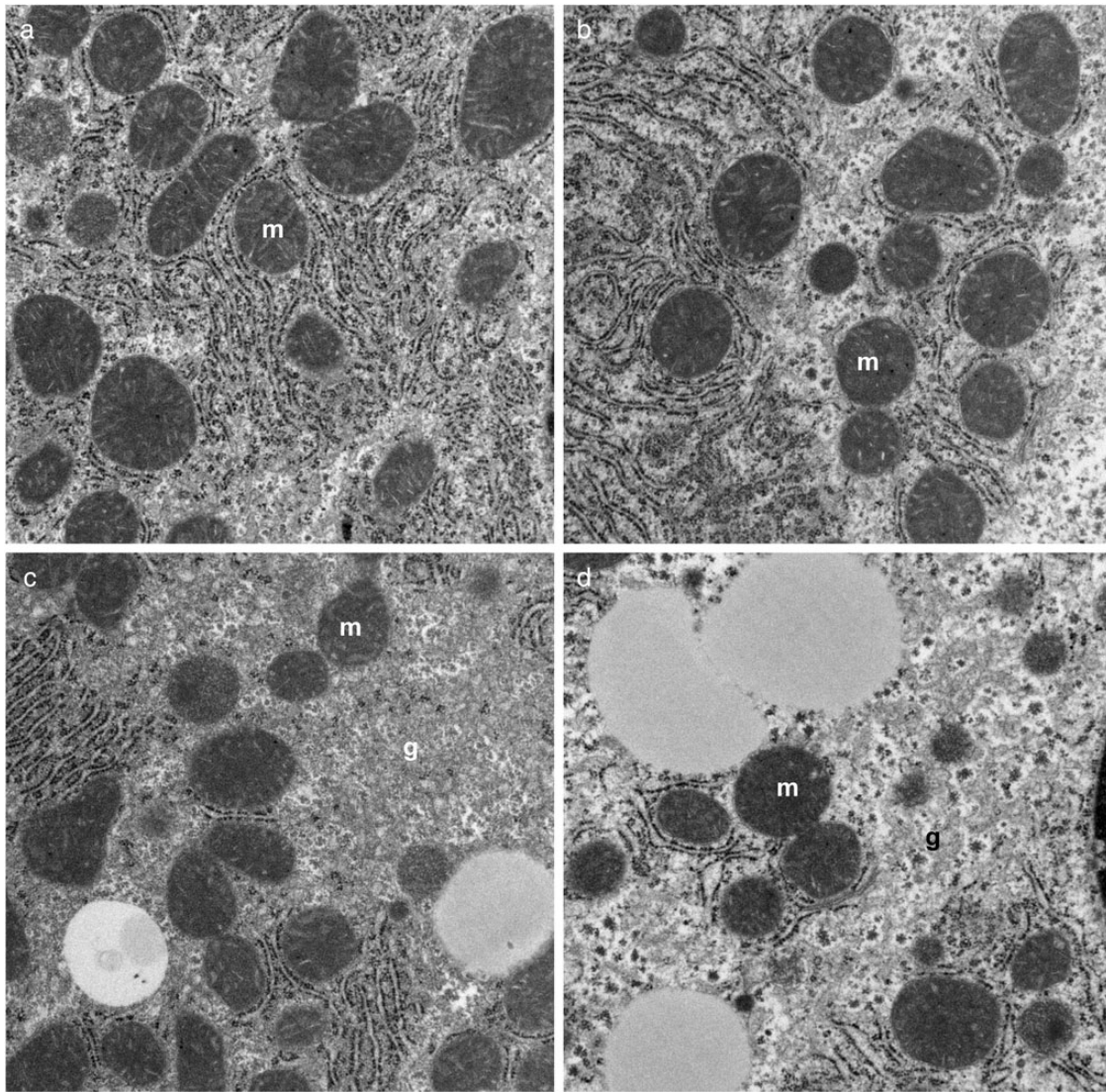


Fig. 3B. (b) NNK, (c), and most strikingly (d) ethanol+NNK decreased the intimate associations between mitochondria (m) and RER relative to (a) control. Note reductions in normal parallel stacks of RER in hepatocytes of the treated groups (original magnification, $\times 18,000$).

significant trends for phosphorylated IRS-1 and relative levels of phosphorylated IGF-1 receptor. In contrast, the effects of NNK on phosphorylated and relative phosphorylated protein levels were less broad and observed with respect to phosphorylated IGF-1 receptor, and relative phosphorylation of the insulin receptor, IGF-1 receptor and Akt. Ethanol \times NNK interactive effects were minimal; only trends were measured for phosphorylated and relative phosphorylated levels of IRS-1.

Insulin receptor expression was progressively reduced in livers exposed to NNK, followed by ethanol, and then ethanol+NNK. *Post hoc* tests demonstrated these progressive differences to be statistically significant (Fig. 5A). In contrast, IGF-1 receptor (Fig. 5B) and Akt (Fig. 5D) expression did not significantly differ among the groups. IRS-1 immunoreactivity was similar in vehicle and NNK treated control livers, but significantly elevated by ethanol exposure, irrespective of NNK treatment (Fig. 5C). GSK-3 β immunoreactivity was significantly elevated in all experimental groups relative to control (Fig. 5E). p70S6K immunoreactivity was similar among the four groups, although a small

difference was detected between the ethanol and ethanol+NNK groups (Fig. 5F).

Ethanol feeding significantly inhibited phosphorylation of the insulin receptor (Fig. 6A), Akt (Fig. 6D), GSK-3 β (Fig. 6E), p70S6K (Fig. 6F), and PRAS40 (Fig. 6G), and combined ethanol+NNK exposures either modestly or significantly reduced phosphorylated levels of insulin receptor (Fig. 6A), IGF-1 receptor (Fig. 6B), IRS-1 (Fig. 6C), and Akt (Fig. 6D) relative to ethanol treatment alone. In contrast, NNK exposure alone had no significant effect on protein phosphorylation relative to control.

Relative levels of insulin receptor phosphorylation were significantly higher in ethanol treated relative to control livers (Supplementary Fig. S5A). However, this effect was due to the greater magnitude in which insulin receptor expression was inhibited compared with the degree of phosphorylation. In contrast, ethanol exposure significantly reduced relative phosphorylation levels of IRS-1 (Supplementary Fig. S5C), Akt (Supplementary Fig. S5D), GSK-3 β (Supplementary Fig. S5E), and p70S6K (Supplementary Fig. S5F), and NNK (with

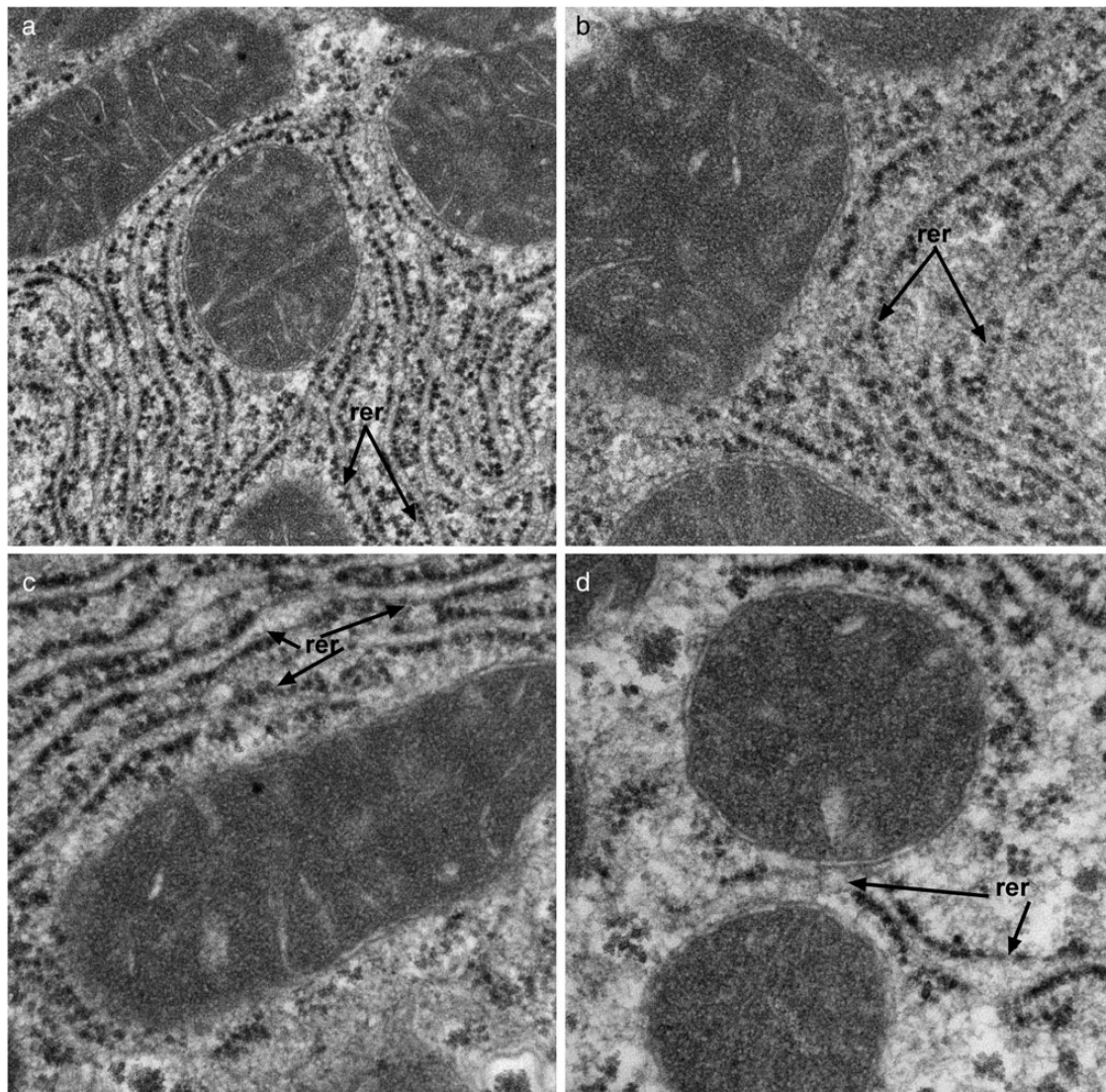


Fig. 3C. Disrupted RER (rer; arrows) morphology with irregular loss of ribosomes and dilations of the normally parallel ER tubules in the (b) NNK, (c) ethanol, and (d) ethanol+NNK exposed hepatocytes relative to control (a) (original magnification, $\times 44,000$).

or without ethanol) reduced relative phosphorylation of the IGF-1 receptor (Supplementary Fig. S5B), Akt (Supplementary Fig. S5D), and GSK-3 β (Supplementary Fig. S5E).

DISCUSSION

Chronic plus binge ethanol and tobacco nitrosamine (NNK) related liver disease

The chronic plus binge ethanol exposure model was used to mimic the human clinical scenario that leads to alcoholic hepatitis. A second goal was to examine NNK tobacco nitrosamine effects on liver injury, steatohepatitis and hepatic insulin resistance, in light of the facts that: (a) a very high percentage of heavy drinkers also smoke (Romberger and Grant, 2004); (b) the causes of progressive alcoholic hepatitis are more complex than alcohol consumption *per se*; (c) previous studies showed that chronic exposure to sub-mutagenic levels of other nitrosamines causes steatohepatitis (de la Monte *et al.*, 2009b); and (d) NNK causes oxidative injury and DNA damage in

liver due to formation of *O*⁶-methyl-Gua, pyridyloxobutyl-, and pyridylhydroxybutyl-DNA adducts (Lao *et al.*, 2007; Upadhyaya *et al.*, 2009). Our hypothesis is that the tobacco nitrosamines specifically mediate liver disease that overlaps with and exacerbates the adverse effects of alcohol. The rationale for studying effects of NNK rather than tobacco smoke is that smoking could confound the results by causing pulmonary disease (Gan *et al.*, 2011; McCaskill *et al.*, 2011).

In our ethanol, NNK and ethanol+NNK models, we detected various alterations in serum chemistry. Reduced serum cholesterol and albumin are common findings in heavy drinkers with liver disease, and alcohol-associated increases in anion gap reflect metabolic acidosis. On the other hand, the reduced or unchanged levels of ALT, LDH, AST and alkaline phosphatase were unexpected and discordant with conventional wisdom since acute hepatocellular injury usually causes high liver enzyme levels in serum. Moreover, the data conflict with previous observations in chronic ethanol (Denucci *et al.*, 2010) and chronic+binge ethanol (Miller *et al.*, 2011; Bertola *et al.*, 2013a,b) exposure models. To account for these discrepant

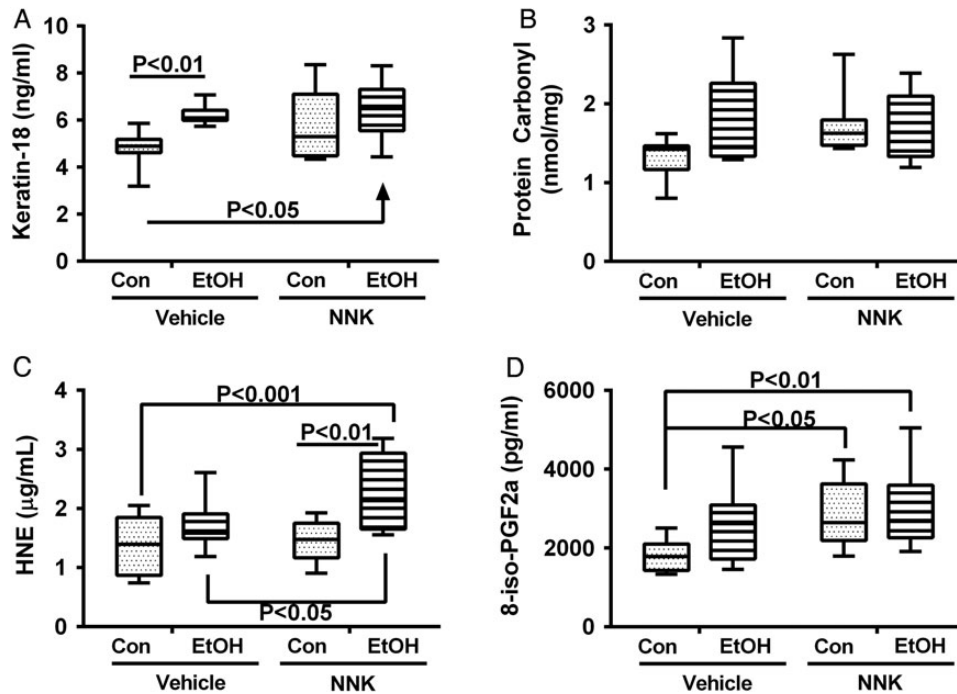


Fig. 4. Ethanol and NNK effects on stress indices in liver. Competitive ELISAs were used to measure (A) cytokeratin 18, (B) protein carbonyl, (C) 4-hydroxy-2-nonenal (HNE) and (D) 8-iso-PGF-2 α , with results normalized to sample protein. Inter-group comparisons were made by two-way ANOVA (Supplementary Table S4). Results of *post hoc* Tukey multiple comparison tests are shown in the panels.

results, we considered two potential explanations: (a) impaired hepatocellular function with deterioration of hepatic machinery paradoxically suppressed serum enzyme levels (Kanno *et al.*, 2000); and (b) the assay results may have been compromised due to minute (non-visible) contamination with free hemoglobin. The authors favor the latter since we presented ample evidence of on-going hepatocellular injury in all three models of steatohepatitis, and several reports have highlighted problems occurring with automated selected enzyme chemistry assays, i.e. LDH and transaminases, due to hemoglobin contamination, even in very small amounts (Lippi *et al.*, 2006; Cameron *et al.*, 2009; Koseoglu *et al.*, 2011). None of our samples had visibly detectable hemolysis. However, in contrast to our previous studies in which the serum assays were performed immediately, locally and manually, in the present study, the serum chemistry assays were performed by a commercial laboratory using automated equipment.

In humans, ALD is associated with hepatic steatosis, acute inflammation, necrosis, ballooning degeneration of hepatocytes and disorganization of the lobular architecture (Tannapfel *et al.*, 2011; Yerian, 2011). In the present study, livers from all three experimental groups exhibited steatosis, subacute inflammation, hepatocellular apoptosis or necrosis, and disruption of the chord-like architecture, and therefore mimicked many aspects of human ALD. In contrast to the reports by Bertola *et al.* (2013a), we did not detect neutrophil infiltration in our chronic+binge ethanol exposure models. This difference could be explained by the higher binge ethanol dose (5 g/kg) used compared with the present experiment (2 g/kg).

Although the NNK-induced steatohepatitis was not as severe as with ethanol, it was worse than with other nitrosamine exposures, i.e. NDEA and streptozotocin (Tong *et al.*, 2009, 2010; Jin *et al.*, 2010), and the features over-lapped with

ALD. Moreover, NNK's adverse effects on steatohepatitis appeared to be additive with ethanol, particularly with regard to the severity of fibrosis (Sirius red staining), which marks ALD progression, and ultrastructural pathology manifested by mitochondrial depletion and RER disruption. These findings suggest that chronic exposure to submutagenic nitrosamines present in tobacco smoke can cause steatohepatitis, worsen alcoholic liver disease and possibly enhance ALD progression. Mechanistically, NNK-induced steatohepatitis is likely mediated by NNK metabolism (Atalla *et al.*, 2000) and DNA adduct formation (Lao *et al.*, 2006; Upadhyaya *et al.*, 2008; Stepanov and Hecht, 2009) in liver. Correspondingly, we detected *O*⁶-methyl-Gua in the NNK-exposed livers. It is not clear why blood alcohol concentrations were lower in the ethanol+NNK relative to ethanol-only exposures. We speculate that competition for cytochrome P450 enzymes may play a role. For example CYP2E1 is induced by alcohol, generating oxidative radicals that cause cell injury (Lieber, 2004; Bardag-Gorce *et al.*, 2006), and also activating NNK (Yue *et al.*, 2009) which can alter various enzyme activities.

Chronic plus binge alcohol and NNK effects on inflammatory and stress mediators of ALD

The multiplex ELISA results demonstrated broad activation of pro-inflammatory cytokines in all experimental groups. This result contrasts with the relatively modest levels of cytokine activation observed previously in chronic ethanol exposure models (Setshedi *et al.*, 2011; Ramirez *et al.*, 2013). However, the increased levels of TNF- α and IL-1 β with ethanol exposures are consistent with previous reports in other studies of ALD (Ronis *et al.*, 2005, 2010; Singh *et al.*, 2007; Bertola *et al.*, 2013b). However, the independent effects of NNK with

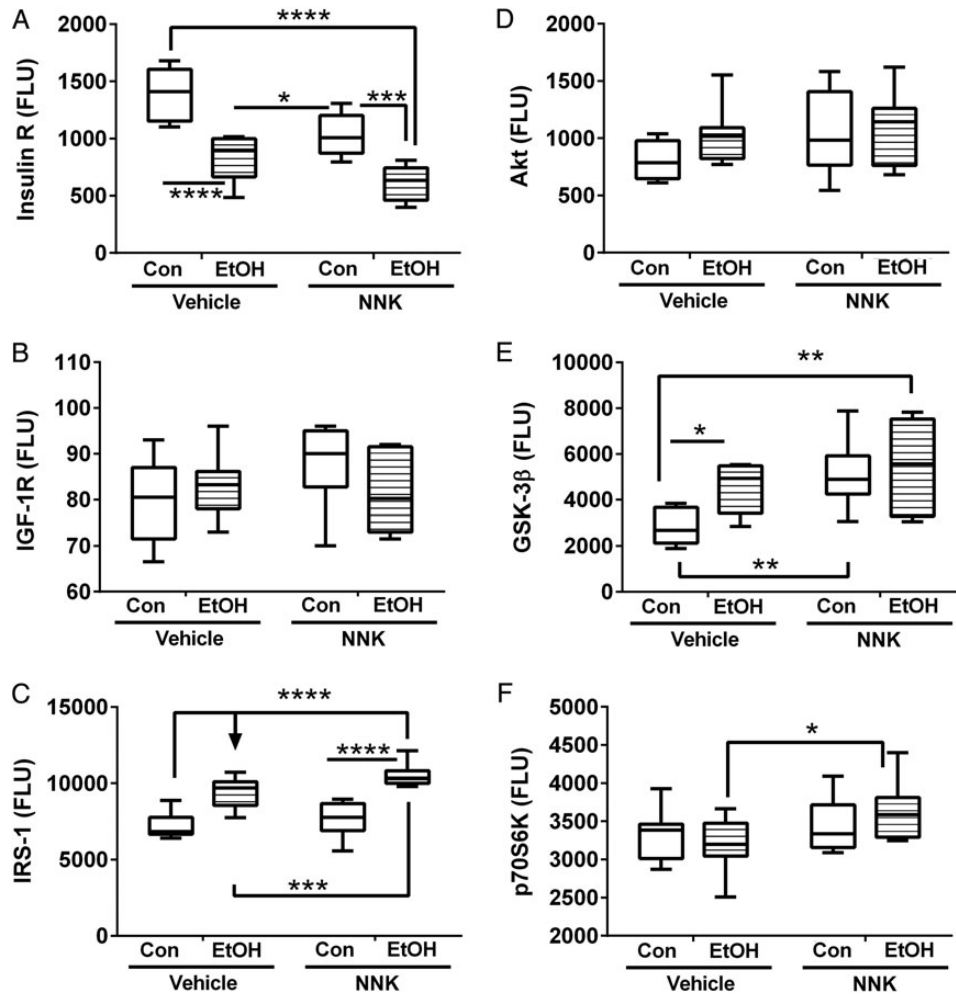


Fig. 5. Ethanol and NNK effects on hepatic expression of insulin/IGF-Akt pathways signaling proteins in liver. Liver protein homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to (A) insulin receptor (Insulin R), (B) IGF-1 receptor (IGF-1R), (C) IRS-1, (D) Akt, (E), GSK-3 β , and (F) p70S6K. Data were analyzed by two-way ANOVA (Supplementary Table S5), and results from *post hoc* Tukey multiple comparison tests are shown in the panels (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

respect to IL-6 and IL-1 β activation are novel. Given their separate effects on inflammation and cytokine activation, the lack of additive ethanol+NNK exposure effects was somewhat surprising since co-administration of ethanol and nitrosamines was previously shown to sharply increase *O*⁶-methyl-Gua DNA adduct formation (Chhabra et al., 2000). Nonetheless, the key point is that both ethanol and tobacco nitrosamine exposures cause chronic pro-inflammatory states in liver, and thereby contribute to on-going hepatocellular injury and cell death. Since inflammation worsens insulin resistance, oxidative stress, and dysregulated lipid metabolism, chronic, broad activation of pro-inflammatory mediators due to ethanol and/or low-level tobacco nitrosamine exposures are likely to contribute to ALD pathogenesis and progression.

Besides the activation of pro-inflammatory mediators, NNK and alcohol cause oxidative stress, DNA damage and adduct formation (Bhagwat et al., 1998; Upadhyaya et al., 2008; Pang et al., 2009; Stepanov and Hecht, 2009; Setshedi et al., 2011; Ramirez et al., 2013). Some of these effects are mediated through regulation of cytochrome P450 (CYP) genes and enzymes. For example, nicotine stimulates expression of the CYP2A6 and CYP1A1 which promote oxidative stress due to metabolism of

nicotine to NNK and cotinine (Ande et al., 2012). CYP2A6, CYP2A13, CYP2B1 and CYP1A2 promote NNK activation via alpha-hydroxylation (Guo et al., 1991; Smith et al., 1996; Chiang et al., 2011). Ethanol induces CYP2E1 (Lieber, 2004; Bardag-Gorce et al., 2006) and CYP2B1/2 (Yue et al., 2009), which enhance NNK activation (Guo et al., 1991; Smith et al., 1995; Ardies et al., 1996). Activated NNK methylates and pyridyloxobutylates DNA (Peterson et al., 1993); NNK methylation of DNA is marked by the formation of *O*⁶-methyl-Gua adducts (Hecht et al., 1993; Chiang et al., 2011). In addition, NNK and other tobacco-specific nitrosamines can form adducts with proteins, such as globin (Carmella and Hecht, 1987; Hecht et al., 1991, 1994; Upadhyaya et al., 2009).

Beside causing permanent macromolecular injury including DNA damage (Becher et al., 1993; Wang et al., 2012b), NNK adducts can alter gene expression (Devereux et al., 1993) by interacting with or methylation of DNA promoter regions (Cloutier et al., 1999; Pulling et al., 2001, 2004; Vuilleminot et al., 2006; Kimura et al., 2012; Wang et al., 2012a). In addition, CYP2E1, together with alcohol dehydrogenase and catalase, oxidizes alcohol to acetaldehyde, which is toxic and leads to adduct formation on proteins, nucleic acids and lipids

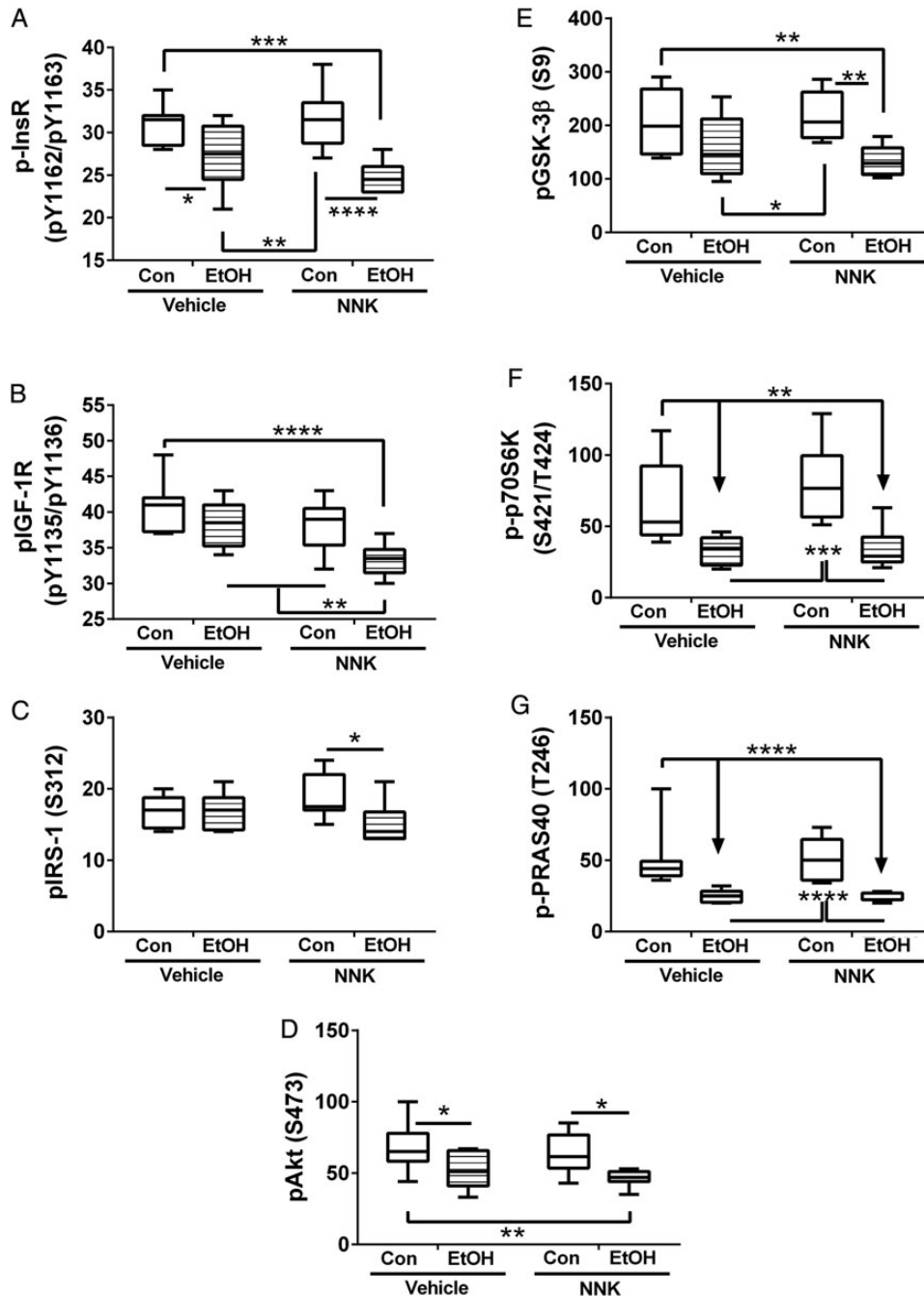


Fig. 6. Ethanol and NNK effects on insulin/IGF-Akt pathway activation in liver. Liver protein homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to (A) pYpY1162/1163-Insulin receptor (p-InsR), (B) pYpY1135/1136-IGF-1R, (C) pS312-IRS-1, (D) pS473 AKT, (E) pS9-GSK-3 β , (F) pTpS421/424-p70S6K and (G) pT246-PRAS40. Data were analyzed by two-way ANOVA (Supplementary Table S5), and results from *post hoc* Tukey multiple comparison tests are shown in the panels (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

(Setshedi *et al.*, 2011), unless efficiently metabolized to acetate and water by aldehyde dehydrogenase 2 (Yu *et al.*, 2010). Complete metabolism of alcohol in the presence of NNK may be limited since NNK causes hepatotoxic effects of ethanol and NNK can be manifested by cellular injury and death, mitochondrial dysfunction, disruption of the cytoskeleton, and lipid and protein adduct accumulation.

Cytokeratin 18 marks hepatocellular injury with pre-Mallory inclusions in ALD (Savolainen *et al.*, 1994), and can serve as a non-invasive serum biomarker for hepatocellular injury and death

following toxin exposure (Cave *et al.*, 2011), NAFLD (Smith and Adams, 2011) and heavy alcohol misuse (Gonzalez-Quintela *et al.*, 2011; Lavallard *et al.*, 2011). Carbonylation is a marker of protein oxidation that reflects stress and is elevated in steatohepatitis (Videla *et al.*, 2004). HNE is a dominant aldehydic product of lipid peroxidation of membrane polyunsaturated fatty acids that exerts cytotoxic effects, including via post-translational modification and impairment of ALDH function (Song *et al.*, 2011). In addition, human heavy drinkers with ALD were found to have elevated serum levels of anti-HNE, suggesting host immune

mediated injury contributes to liver disease (Mottaran *et al.*, 2002). Isoprostane 8-iso-PGF₂ α is formed by free radical-catalyzed peroxidation of arachidonic acid independent of cyclooxygenase and therefore marks lipid peroxidation (Morrow *et al.*, 1992, 1994; Sodergren *et al.*, 2000; Yin *et al.*, 2007). Isoprostane levels along with upregulated mRNA expression of lipoxygenases have been observed in human livers with ALD or NAFLD (Raszeja-Wyszomirska *et al.*, 2012). Nitrosylation can impair function of alcohol metabolizing enzymes, including aldehyde dehydrogenase, and thereby exacerbate the toxic effects of ethanol due to acetaldehyde buildup (Moon *et al.*, 2007). Altered protein and lipid structures have been recognized as important features of ALD for decades (Takahashi *et al.*, 1991; Rouach *et al.*, 1997). Our studies showed that ethanol and NNK each increased lipid adducts, although the indices of lipid peroxidation differed in that 8-iso-PGF-2 α was driven more by NNK exposures, while HNE was increased by ethanol, with or without NNK co-exposures. Although hepatic protein carbonylation was not significantly increased by ethanol or NNK, the intra-group variances were high. Future studies will be directed toward examining adduct formation on specific rather than global proteins, as the consequences may be relevant to the observed impairments in liver function.

Chronic plus binge alcohol and NNK effects on hepatic insulin/IGF-1 signaling mediators

Previous studies demonstrated significant impairments in insulin signaling in both experimental models and humans with ALD (Onishi *et al.*, 2003; Ronis *et al.*, 2007; de la Monte *et al.*, 2008; Denucci *et al.*, 2010; Setshedi *et al.*, 2011; Longato *et al.*, 2012; Ramirez *et al.*, 2013). Insulin resistance was linked to three key pathogenic factors: steatosis with dysregulated lipid metabolism; inflammation, and oxidative and ER stress (de la Monte *et al.*, 2009a; Longato *et al.*, 2012; Ramirez *et al.*, 2013). However, since impairments in insulin signaling occur even after short durations of ethanol exposure, they could be regarded as the initiating event, although once established, ALD treatment would likely have to be multipronged to interrupt the cascade of progressive degeneration (de la Monte *et al.*, 2009a; de la Monte, 2012). In our consideration of the potential role of tobacco nitrosamines as mediators or co-factors in ALD, we evaluated effects of NNK on hepatic insulin/IGF-1 signaling mechanisms.

We measured total and phosphorylated proteins involved in signaling through Akt since Akt has a key role in regulating cell growth, survival and metabolism, including lipid homeostasis in liver (Roberts *et al.*, 2000; Li *et al.*, 2005; Michl and Downward, 2005; Leavens and Birnbaum, 2011). The studies showed that both ethanol and NNK impaired expression of the insulin receptor and that the effects were additive. Reduced insulin receptor expression could contribute to impairments in insulin signaling in the liver by limiting functional activation of downstream pathways. In contrast, ethanol mainly was responsible for inhibiting phosphorylation of the insulin receptor, and phosphorylation of the insulin receptor, Akt, and GSK-3 β , but in some instances, e.g. IRS-1, NNK co-exposures further impaired phosphorylation of signaling molecules. However, both ethanol and NNK exposures contributed significantly to the relative reductions in phosphorylation of the IGF-1 receptor (utilized for cellular proliferation and regeneration), Akt and GSK-3 β . Decreased total and relative levels of GSK-3 β

phosphorylation and increased levels of GSK-3 β protein occurred with ethanol or NNK, indicating that both contributed to increased GSK-3 β activation. Importantly, GSK-3 β activation opposes the positive effects of PI3K-Akt signaling and stimulation of growth, metabolism, and cell survival, and its inhibition of oxidative stress, inflammation, and cell death (apoptosis) (Pap and Cooper, 1998; Pearl and Barford, 2002; McCubrey *et al.*, 2014).

Conclusions

These studies provide new evidence that chronic, low-dose (sub-mutagenic) NNK tobacco nitrosamine exposures cause liver injury with molecular, biochemical and histopathological features that overlap with the effects of alcohol, and suggest that they can contribute to the pathogenesis of ALD. Although both ethanol and NNK caused hepatic inflammation, oxidative stress, steatosis and impairments in insulin/IGF-1 signaling through Akt, the characteristics and severities of these effects were distinctive. The most prominent distinguishing effects of ethanol were to inhibit insulin signaling at multiple levels of the cascade, cause severe hepatic steatosis and increase lipid peroxidation, whereas the main effects of NNK were to impair mitochondrial function and increase oxidative injury, GSK-3 β activation and nitrotyrosine adducts. Future studies will determine the degree to which the observed effects of NNK are mediated by O⁶-methyl-Gua adducts with attendant increased DNA damage (genomic and mitochondrial), inhibition of alcohol metabolizing enzymes with increased acetaldehyde accumulation, and increased ER stress with impairment of the ubiquitin-proteasome pathway.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Alcohol and Alcoholism* online.

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Conflict of interest statement. None declared.

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