Published in final edited form as:

Alcohol. 2013 August ; 47(5): 353–357. doi:10.1016/j.alcohol.2013.04.004.

Nicotine enhances ethanol-induced fat accumulation and collagen deposition but not inflammation in mouse liver

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Abstract

Introduction—Alcohol and tobacco are frequently co-abused. Tobacco smoke increases alcoholic steatosis in apoE(−/−) mice. Tobacco smoke contains more than 4000 chemicals, but it is unknown which compounds in tobacco smoke play a major role in increasing alcoholic steatosis.

Methods—C57BL/J6 mice were intraperitoneally injected with nicotine 1 mg/kg every day or saline at the same volume as a control when the mice were fed dextrose-control or ethanol Lieber-DeCarli liquid diets. Three weeks later the mice were sacrificed after overnight fasting.

Results—Neither nicotine injection nor ethanol feeding alone increased serum levels of triglyceride, but the combination of nicotine and ethanol increased serum levels of triglyceride. Both nicotine injection and ethanol feeding alone increased hepatic collagen type I deposition, and nicotine injection and ethanol feeding combined further increased hepatic collagen type I deposition. The combination of nicotine and ethanol also activated hepatic stellate cells, a principal liver fibrogenic cell. Hepatic fat accumulation was induced by ethanol feeding, which was enhanced by nicotine injection. Ethanol feeding caused an increase in serum ALT, but nicotine did not further increase serum ALT levels. Lipid droplets and inflammatory foci were observed in liver sections from ethanol-fed mice; nicotine treatment increased the number and size of lipid droplets, but not the number and size of inflammatory foci. Nicotine did not further increase ethanol-induced hepatic neutrophil infiltration.

Conclusions—These results suggest that nicotine enhances ethanol-induced steatosis and collagen deposition, but nicotine has no effect on ethanol-induced inflammation.

Keywords

CYP2A5; ethanol; nicotine; steatosis; collagen

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Declaration of Interests: The authors declare no conflict of interest to each other.

INTRODUCTION

Chronic alcohol abuse causes many disorders including alcoholic liver disease (ALD), which ranges from fatty liver (steatosis), steatohepatitis (inflammation) to liver fibrosis and cirrhosis, even liver cancer. Alcohol-induced fatty liver (alcoholic steatosis) is seen in more than 90% of alcoholic patients (Lieber, 2005; Lu and Cederbaum, 2008). Exposure to cigarette smoke predisposes to cardiovascular and lung diseases and several types of cancer (Thun et al., 2000). Some evidence suggests the association of cigarette smoke with increased severity of hepatic fibrosis in patients with primary biliary cirrhosis (Zein et al., 2006). Alcohol and tobacco are frequently co-abused (Sellers et al., 2003). Tobacco smoke increases alcoholic steatosis in apoE(−/−) mice (Bailey et al., 2009). Tobacco smoke contains more than 4000 chemicals, but it is unknown which compounds in tobacco smoke play a major role in increasing alcoholic steatosis. Nicotine is a major component of tobacco smoke. Nicotine exerts its cellular functions in neuronal cells through nicotinic acetylcholine receptors (nAChRs) (Itier and Bertrand, 2001). Cotinine, a metabolite of nicotine, has much lower potency to bind to nAChR (Vainio and Tuominen, 2001). CYP2A6 is a major enzyme which metabolically inactivates nicotine in humans, while in mice the enzyme which inactivates nicotine is CYP2A5, a mouse ortholog of human CYP2A6 (Su and Ding, 2004). It has been reported that human hepatic CYP2A6 expression is increased in alcoholic patients (Niemela et al., 2000). Recently we observed an increase in CYP2A5 in mice after ethanol feeding (Lu et al., 2011, 2012).

Ethanol exposure induces lipolysis in white adipose tissue and release of free fatty acids, which in turn promotes fatty liver (Horning et al., 1960; Zhong et al., 2012). Smoking also stimulates lipolysis *in vivo* via nicotine-mediated release of catecholamines (Kershbaum et al., 1963). In addition, adipocytes also express nAChRs, and nicotine can stimulate adipocytes to release free fatty acids (Liu et al., 2004). Alcohol can also induce liver fibrosis and cirrhosis, and activate hepatic stellate cells (HSCs) to become major fibrogenic cells in the liver (Friedman, 2008). Nicotine can stimulate HSC proliferation and collagen secretion via nAChRs (Soeda et al., 2012). We hypothesize that nicotine stimulates lipolysis in adipose tissue to enhance ethanol-induced fat accumulation in the liver, and that nicotine enhances ethanol-induced liver fibrosis via activation of HSCs.

MATERIALS AND METHODS

Animals and Treatment

C57BL/6 background male WT mice (purchased from Charles River Laboratory) were housed in temperature-controlled animal facilities with 12-hour light/12-hour dark cycles and were permitted consumption of tap water and Purina standard chow *ad libitum* until being fed the liquid diets. The mice received humane care and experiments were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and with approval of the Mount Sinai Animal Care and Use Committee.

All mice were initially fed the control liquid-dextrose diet (Bio-Serv, Frenchtown, NJ) for 3 days to acclimate them to the liquid diet. Afterward, the mice were fed either the liquid ethanol diet (Bio-Serv, Frenchtown, NJ) or the control liquid-dextrose diet for 3 weeks (Lu et al., 2008, 2010). The content of ethanol was increased every 3 days from 10% (1.77% [vol/vol]) of total calories to 20% (3.54% [vol/vol]), 25% (4.42% [vol/vol]), 30% (5.31% [vol/vol]), and finally 35% (6.2% [vol/vol]) of total calories. The control mice were pair-fed the dextrose diet on an iso-energetic basis. The ethanol-fed mice had access to their ration *ad libitum*. During the 3 weeks of feeding with ethanol or dextrose liquid diets, nicotine hydrogen tartrate salt was injected intraperitoneally daily at 1 mg/kg body weight or saline

at the same volume as a control. Nicotine can reduce food intake (Mineur et al., 2011), but no decrease in food consumption was observed in our model.

The mice were sacrificed by cervical dislocation after blood was collected under anesthesia via the retro-orbital venous sinus. The liver was removed intact and liver weight was measured; then the liver was rapidly excised into fragments and washed with cold saline, and one aliquot of tissue was placed into 10% formalin solution for paraffin blocking. The remaining aliquots were stored at -80°C for further assays. Liver homogenates were prepared in ice-cold 0.15M potassium chloride.

Liver Histology and Immunohistochemistry (IHC)

Liver sections were stained with hematoxylin-eosin (H&E) for pathological evaluation. Steatosis was quantified as the percentage of cells containing fatty droplets. Necro-inflammation was quantified as the number of clusters of 5 or more inflammatory cells per square millimeter (one $200 \times$ magnification field area = $.95 \text{ mm}^2$). The pathologist (Dr. Stephen Ward) was unaware of the treatment groups when evaluating the slides.

IHC for collagen type I and α HC for muscle actin (muscle was performed by using anti-collagen type I antibody (Millipore) and anti- α -SMA antibody (Abcam), followed by a Broad Spectrum (AEC) Histostain-Plus kit (Invitrogen). No staining was observed in the absence of the primary antibody. Naphthol AS-D chloroacetate esterase (Sigma) was used for *in situ* identification of neutrophils.

Liver Triglyceride (TG), Serum Alanine Aminotransferase (ALT), Ethanol and TG

Liver and serum TG, serum ALT, and ethanol were assayed using kits from Pointe Scientific (Canton, MI), respectively.

Cytochrome P450 2E1 and 2A5 Activity in Hepatic Microsomes

Hepatic microsomes were prepared as we described previously (Lu et al., 2005). CYP2E1 activity was measured by the rate of oxidation of 1mM *p*-nitrophenol to *p*-nitrocatechol by 100 μm of microsomal protein for 15 minutes at 37°C (Lu et al., 2005). CYP2A5 activity was measured by assessing coumarin 7-hydroxylase activity with 100 μC coumarin as substrate plus 100 μC of microsomal protein and incubation for 15 minutes at 37°C (Lu and Cederbaum, 2006).

Western Blotting

Hepatic proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After 1 hour of blocking with 2% fat-free milk, membranes were then incubated overnight with rabbit anti-CYP2E1 IgG (a gift from Dr. Jerome Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ), chick anti-CYP2A5 IgG (a gift from Dr. Risto Juvonen, Department of Pharmacology and Toxicology, University of Kuopio, Kuopio, Finland), and rabbit anti-alcohol dehydrogenase (ADH), goat anti-calnexin (Santa Cruz), and mouse anti-collagen type I (Millipore), followed by 1 hour incubation with peroxidase secondary anti-rabbit, anti-chick, anti-mouse or anti-goat antibodies (Millipore), respectively. Chemiluminescence was detected by Image Reader LAS-4000 (Fujifilm) after adding Pierce EC Western Blotting Substrate (Thermo Scientific). Blots were quantified using the ImageJ (version 1.37) software program from the National Institutes of Health. Ratios were expressed as protein/calnexin.

Statistics

Results are expressed as means $\pm$ SEMs. Statistical evaluation was carried out by one-way ANOVA followed by the Student-Newman-Keuls *post hoc* test. A value of $p < .05$ was considered as statistical significance.

RESULTS

Nicotine treatment enhances ethanol-induced steatosis but does not affect ethanol-induced necro-inflammation

Neither control-diet feeding alone nor nicotine treatment alone induced a necro-inflammatory response. After 3 weeks of ethanol feeding, small foci of necro-inflammation were observed in liver sections, but nicotine did not increase the number or size of necro-inflammatory foci (Fig. 1A **H&E staining**; Fig. 1B). Ethanol-induced neutrophil infiltration in the liver (indicated by NASDCA staining) was not further increased by nicotine administration (Fig. 1A **NASDCA staining**). Consistently, after ethanol feeding, serum levels of ALT were increased about 2-fold compared with control-diet feeding, but nicotine treatment did not further increase serum ALT levels (Fig. 1D). These results suggest that nicotine did not enhance ethanol-induced liver injury.

Neither control-diet feeding nor nicotine injection induced steatosis (Fig. 1A **H&E staining**; Fig. 1C). As expected, after 3 weeks of ethanol feeding, lipid droplets were observed in liver sections with H&E staining and nicotine injection increased the number and size of lipid droplets (Fig. 1A **H&E staining**; Fig. 1C), suggesting that nicotine enhanced ethanol-induced steatosis. Consistently, hepatic TG levels were increased in mice fed the ethanol diet compared to mice fed the control diet, and nicotine injection further increased ethanol-induced TG accumulation in the liver (Fig. 1E). Neither ethanol feeding nor nicotine injection increased serum levels of TG, but the combination of nicotine and ethanol caused an increase in serum TG by 2-fold (Fig. 1F). These results suggest that nicotine enhances ethanol-induced steatosis.

Nicotine treatment during ethanol feeding increases hepatic collagen type I deposition

To see if nicotine enhances alcohol-induced collagen deposition (fibrosis), hepatic contents of collagen type I were detected. When the mice receiving the control diet were injected with nicotine, hepatic contents of collagen type I were increased (Fig. 2A). When the mice were fed ethanol without nicotine, they also showed increases in collagen type I. Collagen type I was further increased by the combination of nicotine injection and ethanol feeding (Fig. 2A). IHC showed that collagen type I was mainly distributed in sinusoids around central veins, and distribution of collagen type I was extended from central veins to portal veins by the combination of nicotine injection and ethanol feeding (Fig. 2B). HSC activation was detected by α -SMA. As shown in Figure 2C, although walls of blood vessels are positive for α -SMA in all groups, isolated single-cell staining was observed only in the group of mice that received nicotine plus ethanol. Ethanol feeding or nicotine injection did not significantly change expression of ADH (Fig. 2A), and nicotine administration did not affect blood ethanol levels (Fig. 1G), suggesting that nicotine-enhanced steatosis and collagen type I deposition are not due to changes in ethanol metabolism.

Nicotine treatment enhances ethanol-induced CYP2A5 but not CYP2E1

Chronic ethanol can induce CYP2E1 and CYP2A5 enzyme expression, and the ethanol induction of CYP2A5 expression is CYP2E1-dependent (Lu et al., 2011, 2012). In mice, CYP2A5 is a major nicotine-metabolizing enzyme in the liver (Su and Ding, 2004). Expression and activity of CYP2E1 and CYP2A5 were examined. Ethanol alone or nicotine

alone induced CYP2A5 protein expression (Fig. 2A) and activity (Fig. 2D), and the combination of ethanol and nicotine had an additive effect on CYP2A5 induction (Figs. 2A, 2D). However, nicotine did not induce CYP2E1 expression; nicotine did not enhance ethanol induction of CYP2E1, either (Figs. 2A, 2D).

DISCUSSION

In this study we found that during ethanol feeding, injection of nicotine, a major component of tobacco smoke, increased serum levels of TG and further increased hepatic collagen type I deposition and fat accumulation but not serum ALT levels. In liver sections, number and size of lipid droplets but not inflammatory foci induced by ethanol were increased by nicotine injection. These results suggest that nicotine enhances ethanol-induced steatosis and hepatic collagen deposition, but nicotine has no effect on ethanol-induced inflammation in the liver. Nicotine administration did not affect blood ethanol levels, suggesting that nicotine-enhanced steatosis and collagen type I deposition are not due to changes in ethanol metabolism.

As a major component of tobacco smoke, nicotine is mainly absorbed via the lungs. In this study, we injected nicotine intraperitoneally, which is clearly different from human ingestion of nicotine. The advantage of injecting nicotine in animal studies is that it is easy to administer and the dose can be precisely controlled. Commercially available nicotine can be either a free base liquid (MW 162) or in the form of a tartrate salt. We used hydrogen tartrate salt with an anhydrous molecular weight of 462 at 1 mg/kg. To determine the amount of free base nicotine present in a nicotine bitartrate salt, multiply the mass of the nicotine bitartrate salt by 162/462, or 0.35. This shows that our dose of free base nicotine was 0.35 mg/kg body weight. In human smokers, urine cotinine, a major metabolite of nicotine and a marker of nicotine exposure, ranges from 1500 ng/mL to 8000 ng/mL (Matta, et al., 2007). Mice receiving 1 mg/kg nicotine intraperitoneally 3 times per week had an average cotinine of 3000 ng/mL in their urine (Davies et al., 2009). Nicotine injected intraperitoneally twice per day at 0.75 mg/kg is approximately equivalent to the amount of nicotine exposure a human receives from 2 packs of cigarettes per day (Friedman et al., 2012; Matta, et al., 2007). Therefore, the nicotine exposure of the mice is approximately equivalent to the amount of nicotine absorbed by heavy smokers.

It is well known that alcohol consumption causes liver fibrosis and cirrhosis marked by increased collagen deposition. In the liver, HSCs are the principal fibrogenic cells. HSCs are quiescent under physiological conditions and contain lipid droplets storing retinol. When HSCs are activated, they release lipid droplets and produce and release more collagen (Friedman, 2008). Ethanol may regulate HSC function; increased oxidative stress response and transforming growth factor (TGF) β play an important role in ethanol-regulated type I collagen transcription in HSCs (Wang et al., 2006). Nicotine can also induce oxidative stress in the liver (El-Sokkary et al., 2007; Taysi et al., 2010), which may contribute to activation of HSCs. Usually HSC activation is associated with inflammation (Friedman, 2008). However, in our study nicotine did not enhance ethanol-induced inflammation. Recently it was found that HSCs express nAChRs, and that nicotine induces proliferation of HSCs and expression of collagen type I and TGF β (Soeda et al., 2012). In this study we observed HSC activation only when nicotine and ethanol exposure were combined (Fig. 2C). It is possible that nicotine enhanced hepatic collagen type I deposition and fibrosis through direct activation of HSCs through binding to nAChR.

Nicotine exerts its cellular functions through nAChRs (Itier and Bertrand, 2001). But no evidence shows that hepatocytes express nAChRs (Hiramoto et al., 2008). It was reported that ethanol feeding up-regulates the endocannabinoid 2-arachidonoylglycerol (2-AG) and

its biosynthetic enzyme diacylglycerol lipase beta selectively in HSCs, and that paracrine activation of hepatic CB1 receptors by HSC-derived 2-AG mediates ethanol-induced steatosis (Jeong et al., 2008). HSCs express nAChRs, and nicotine can induce proliferation of HSCs (Soeda et al., 2012). HSCs were activated when nicotine and ethanol were combined (Fig. 2C). It is unknown whether nicotine can also up-regulate 2-AG and its secretion from HSCs. It is possible that nicotine enhances ethanol-induced fatty liver via cell-cell interaction, i.e. nicotine may enhance fat accumulation in hepatocytes by activating HSCs to secrete 2-AG, which in turn activates hepatic CB1 receptors and induces fat accumulation in hepatocytes. In addition, nicotine may enhance fat accumulation in the liver via organ-organ interaction. Ethanol exposure induces lipolysis in white adipose tissue, which was suggested to contribute to the fat accumulation in the liver (Horning et al., 1960; Zhong et al., 2012). Smoking also stimulates lipolysis *in vivo*, and the lipolytic effect of smoking has been attributed to the nicotine-mediated release of catecholamines (Kershbaum et al., 1963). Upon stimulation by catecholamines, perilipin, an adipocyte-specific lipid droplet-associated protein, is heavily phosphorylated by protein kinase A, and the phosphorylated perilipin facilitates lipolytic activity of hormone sensitive lipase and adipose triglyceride lipase (Le Lay and Dugail, 2009). Adipocytes express nAChRs (Liu et al., 2004), so nicotine also has a direct effect on white adipose tissue (Chajek-Shaul et al., 1994; Andersson and Arner, 1995). Adipocytes incubated with nicotine released free fatty acids into the medium in a dose-dependent manner (Liu et al., 2004). Therefore nicotine may enhance ethanol-induced steatosis via organ-organ interaction, i.e. adipose tissue-liver interaction.

Ethanol can induce CYP2E1 and CYP2A5, which contributes to ethanol-induced fatty liver (Lu et al., 2008, 2010). But nicotine did not further increase ethanol-induced CYP2E1. Ethanol can also induce CYP2A5 (Lu et al., 2011, 2012). Although nicotine did not further increase CYP2E1 levels in the liver, nicotine further increased ethanol-induced CYP2A5. Does CYP2A5 play a role in nicotine-enhanced alcoholic steatosis? CYP2A5 is a major enzyme in the liver which metabolizes nicotine (Su and Ding, 2004), and the mouse was considered a good model for nicotine metabolism because mouse CYP2A5 metabolizes nicotine similarly to human CYP2A6 (Raunio et al., 2008). It is possible that nicotine metabolites produced by CYP2A5 enhance alcoholic fatty liver. On the other hand, metabolites of nicotine have a very low potency to bind to nAChRs (Vainio and Tuominen, 2001). Thus the cell-cell or organ-organ interaction as postulated above would be impaired. The role of CYP2A5 in nicotine-enhanced alcoholic steatosis is under investigation in our lab.

Acknowledgments

We thank Dr. Jerome Lasker for CYP2E1 antibody and Dr. Risto Juvonen for CYP2A5 antibody.

Grants: These studies were supported by USPHS grants RO1 AA- 017425 and AA -018790 from the National Institute on Alcohol Abuse and Alcoholism and ABMRF/The Foundation for Alcohol Research.

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Figure 1.

Nicotine enhanced ethanol-induced steatosis but not necro-inflammation. During 3 weeks of ethanol or control-diet feeding, nicotine was injected every day as described in Materials and Methods.

(A) H&E and NASDCA staining. The insets show foci of necro-inflammation. White arrows show lipid droplets and black arrows show positive NASDCA staining (neutrophils). PV = portal vein; CV = central vein

(B) Quantification of necro-inflammation (necrosis scores) as described in Materials and Methods.

(C) Quantification of steatosis (steatosis scores) as described in Materials and Methods.

Serum ALT (A), hepatic TG (B), serum TG (C), and serum alcohol (D) were measured as described in Materials and Methods.

- = control; N = nicotine; E = ethanol; NE = nicotine plus ethanol.

* $p < .05$, compared to -; & $p < .05$, compared to N; # $p < .05$, compared to E.

Figure 2.

The combination of ethanol and nicotine-induced collagen type I deposition and CYP2A5 in liver.

(A) Hepatic collagen type I, ADH, CYP2E1, and CYP2A5 protein expression were determined by Western blotting as described in Materials and Methods. The numbers below the bands are densitometric and statistical analyses.

* $p < 0.05$, compared to Control group; $p < 0.0026$; $p < 0.05$, compared to Nicotine group; $p < 0.0023$; $p < 0.05$, compared to Ethanol group.

(B) Hepatic collagen type I was determined by immunohistochemistry staining as described in Materials and Methods. PV = portal vein; CV = central vein.

(C) α -SMA was detected by immunohistochemistry staining. Arrowheads show positive HSC staining; arrows show positive blood vessel wall staining.

(D) Microsomal CYP2E1 and CYP2A5 activities.

* $p < 0.05$, compared to Control group; $p < 0.0026$; $p < 0.05$, compared to Nicotine group; $p < 0.0023$; $p < 0.05$, compared to Ethanol group.