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Nicotine enhances alcoholic fatty liver in mice: Role of CYP2A5

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Abstract

Tobacco and alcohol are often co-abused. Nicotine can enhance alcoholic fatty liver, and CYP2A6 (CYP2A5 in mice), a major metabolism enzyme for nicotine, can be induced by alcohol. CYP2A5 knockout (*cyp2a5*$^{-/-}$) mice and their littermates (*cyp2a5*$^{+/+}$) were used to test whether CYP2A5 has an effect on nicotine-enhanced alcoholic fatty liver. The results showed that alcoholic fatty liver was enhanced by nicotine in *cyp2a5*$^{+/+}$ mice but not in the *cyp2a5*$^{-/-}$ mice. Combination of ethanol and nicotine increased serum triglyceride in *cyp2a5*$^{+/+}$ mice but not in the *cyp2a5*$^{-/-}$ mice. Cotinine, a major metabolite of nicotine, also enhanced alcoholic fatty liver, which was also observed in *cyp2a5*$^{+/+}$ mice but not in the *cyp2a5*$^{-/-}$ mice. Nitrotyrosine and malondialdehyde (MDA), markers of oxidative/nitrosative stress, were induced by alcohol and were further increased by nicotine and cotinine in *cyp2a5*$^{+/+}$ mice but not in the *cyp2a5*$^{-/-}$ mice. Reactive oxygen species (ROS) production during microsomal metabolism of nicotine and cotinine was increased in microsomes from *cyp2a5*$^{+/+}$ mice but not in microsomes from *cyp2a5*$^{-/-}$ mice. These

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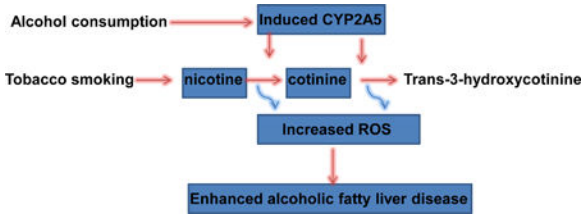
results suggest that nicotine enhances alcoholic fatty liver in a CYP2A5-dependent manner, which is related to ROS produced during the process of CYP2A5-dependent nicotine metabolism.

Graphical Abstract



Keywords

Cotinine; triglyceride; metabolism; oxidative stress; reactive oxygen species; CYP2A6

INTRODUCTION

Alcoholic liver disease (ALD) is a serial of liver disorders caused by heavy alcohol drinking. The spectrum of ALD ranges from hepatic steatosis (fatty liver) to steatohepatitis, fibrosis and cirrhosis (MacSween and Burt 1986). Many of the toxic effects of ethanol have been linked to its metabolism in the liver (Lieber 2005; Lu and Cederbaum 2008). Alcohol dehydrogenase (ADH) is a major metabolic enzyme that is located in cytosol (Lieber 2005). Alcohol is metabolized by ADH to acetaldehyde, which is further metabolized to acetate in mitochondria by acetaldehyde dehydrogenase (ALDH). Compared to ADH-ALDH metabolizing pathway, cytochrome P450 2E1 (CYP2E1) was identified to be a minor metabolic system (Lieber 2005). While ADH activity is decreased after chronic ethanol consumption, CYP2E1 can be induced by chronic ethanol consumption (Lieber 2005). Therefore, CYP2E1 plays an important role in ethanol metabolism during chronic alcohol consumption. Alcoholic fatty liver was developed in wild type (WT) mice but not in the CYP2E1 knockout (*cyp2e1*$^{-/-}$) mice (Lu et al. 2008); in the *cyp2e1*$^{-/-}$ mice reconstituted with human CYP2E1, alcoholic fatty liver was recovered (Lu et al. 2010), suggesting an essential role of CYP2E1 in the development of alcoholic fatty liver. It is generally accepted that CYP2E1 is a risk factor for the development of ALD (Lu and Cederbaum 2008).

In addition to CYP2E1, CYP2A6 (CYP2A5 in mouse) can also be induced by alcohol consumption, and alcohol induction of CYP2A5 was not observed in *cyp2e1*$^{-/-}$ mice although *cyp2e1*$^{-/-}$ mice still express CYP2A5 (Lu et al. 2010, 2012), suggesting that alcohol induction of CYP2A6/5 is CYP2E1 dependent. Unlike CYP2E1, CYP2A5 almost does not metabolize ethanol (Lu et al. 2012). However, CYP2A6/5 is a major enzyme that metabolizes nicotine (Nakajima et al. 1996; Messina et al. 1997; Zhou et al. 2010; Raunio et al. 2008). In mice, about 80% of nicotine is metabolized by CYP2A5 to cotinine (Matta et al. 2007). Nicotine metabolism by CYP2A6/5 can also produce reactive oxygen species (ROS) and induce oxidative stress (Benowitz 2009; Ande et al. 2012).

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Besides alcohol abuse, tobacco cigarette smoking is another worldwide major cause of preventable morbidity and mortality (Knoops et al. 2004). Tobacco smoking induces insulin resistance and elevates plasma triglyceride (TG) (Cena et al. 2011). Tobacco smoke contains more than 4000 chemicals. Nicotine is a major stimulant and addiction-forming alkaloid in tobacco smoke. Pre- and post-natal nicotine exposure might contribute to the development of metabolic disorders later in life (Somm et al. 2009). Alcohol and tobacco are frequently co-abused (Schoedel and Tyndale 2003; Sellers et al. 2003). Epidemiological studies suggest that cigarette smoke exposure may promote alcoholic cirrhosis (Klatsky and Armstrong 1992). Likewise, tobacco smoke exposure can enhance experimental alcoholic fatty liver in apoE knockout mice (Bailey et al. 2005). We found that nicotine can enhance alcoholic fatty liver in mice; while alcohol feeding alone or nicotine injection alone did not affect serum TG, the combination of nicotine and ethanol induces hypertriglyceridemia (Lu et al. 2013).

Alcohol can induce CYP2A5, and CYP2A5 may metabolize nicotine to cotinine and produce ROS, therefore, we hypothesize that CYP2A5 plays an important role in nicotine-enhanced alcoholic fatty liver. In this study, we report that alcoholic fatty liver was enhanced by nicotine in wild type mice but not in CYP2A5 knockout (*cyp2a5*$^{-/-}$) mice, suggesting that CYP2A5 is involved in the enhancing effect of nicotine on alcoholic fatty liver.

METHODS

Materials

Ethanol, nicotine hydrogen tartrate salt, cotinine, potassium chloride (KCL), thiopenbarbituric acid (TBA), trichloride acetic acid (TCA), 2',7'-dichlorofluorescin diacetate (DCF-DA), reduced nicotinamide adenine dinucleotide phosphate (NADPH), p-nitrophenol (PNP) and coumarin were purchased from Sigma-Aldrich, St. Louis, MO, USA. Control dextrose diet powder and ethanol diet powder were purchased from Bio-Serv, Frenchtown, NJ, USA. The liquid ethanol and control diet were prepared by mixing with water with or without ethanol.

Animals

The colony of *cyp2a5*$^{-/-}$ mice was created by crossing male C57BL/6 *cyp2a5*$^{-/-}$ mice (kindly provided by Dr. Xinxin Ding, SUNY College of Nanoscale Science and Engineering, Albany, NY, USA) and female C57BL/6 WT mice (purchased from Charles River Laboratory, MA, USA). Littermates (*cyp2a5*$^{+/+}$) were bred as a colony of WT control. Female, 8–10 weeks old mice were selected for experiment. All the mice 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*. 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. The animal studies were approved by Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai and University Committee on Animal Care at East Tennessee State University, respectively.

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In Vivo administration of ethanol, nicotine, and cotinine

The *cyp2a5*$^{+/+}$ and *cyp2a5*$^{-/-}$ mice were fed the control liquid dextrose diet for 3 days to acclimate them to the liquid diet. Then these mice were either fed the liquid ethanol diet to induce ALD or fed the liquid dextrose diet as a control. Nicotine hydrogen tartrate salt was mixed in the control or ethanol diet at 65 μM (30 mg/L). About 80% of nicotine is metabolized to cotinine, so cotinine was mixed in the liquid diet at 80% x 65 μM=52 μM (9 mg/L). The content of ethanol was gradually increased every 3 days from 10% of total calories (1.77% [vol/vol]) to15% (2.65% [vol/vol]), 20% (3.54% [vol/vol]), 25% (4.42% [vol/vol]), 30% (5.31% [vol/vol]), and finally 35% of total calories (6.2% [vol/vol]). The amount of ethanol diet consumption in the presence or absence of nicotine/cotinine was comparable in *cyp2a5*$^{+/+}$ mice and *cyp2a5*$^{-/-}$ mice. Urine was collected after 2 weeks of feeding. After 18 days of feeding, the mice were sacrificed after a 6 h of fast. Blood was collected for serum biochemical assays. The livers were rapidly excised into fragments and washed with cold saline. Aliquots from same lobes of different mice were put in neutral Formalin solution for paraffin bedding and sectioning. Another aliquot was frozen and cut into frozen sections for oil red O staining. The other liver tissue aliquots were stored at −80°C for future assays.

Liver Histology, immunohistochemistry (IHC), and Oil Red O staining

Paraffin liver sections were used for hematoxylin and eosin (H&E) staining and IHC. Liver sections with H&E staining was used for pathological evaluation as described before (Lu et al. 2008). Malondialdehyde (MDA) and nitrotyrosine (3-NT) were detected by IHC that was performed using anti-MDA (from Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd, Cat#: MMD-030n) and anti-3-NT (from Santa Cruz Biotechnology, Cat#: sc-32757) followed by a Broad Spectrum IHC Select® HRP/DAB kit (from EMD Millipore, Cat#: DAB150). MDA and 3-NT are mainly located around central veins in the liver sections. Quantification for 3-NT and MDA was made by calculating the percentage of positive staining in all central veins.

Frozen liver sections were used for Oil Red O staining as we described before (Lu et al. 2008).

Liver microsomal CYP2E1 and 2A5 Activity.

Liver homogenates were prepared in ice-cold 0.15 M KCL and liver microsomes were extracted as described previously (Lu et al. 2005). Briefly, the homogenate was centrifuged at 9,000 x g for 20 min followed by centrifuging the resulting supernatant fraction at 105,000 x g for 60 min. The microsomes were re-suspended in 0.15 M KCL. All procedures were performed at 4°C. Coumarin is 7-hydroxylated specifically by coumarin 7-hydroxylase (COH) encoded by the mouse cyp2a5 gene and human cyp2a6 gene (Su and Ding 2004). Thus, as a marker of CYP2A5/2A6 catalytic activity, COH activity was evaluated by incubating 100 μg of microsomal protein with 100 μM coumarin and 1 mM NADPH for 15 min at 37 °C in 100 mM potassium phosphate buffer, pH 7.4 (Lu et al. 2010). Fluorescence of 7-hydroxylated coumarin was detected in 390/440 nm. As for CYP2E1 catalytic activity, PNP oxidation was evaluated by incubating 100 μg of microsomal protein with 1 mM PNP

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and 1 mM NADPH for 15 min at 37 °C in 100 mM potassium phosphate buffer, pH 7.4 (Lu et al. 2010). PNP product was detected in 510 nm.

Liver microsomal ROS generation.

Liver microsomal ROS production during nicotine and cotinine metabolism was evaluated by measuring the conversion of DCF-DA to 2',7'-dichlorofluorescein (DCF) (Serron et al. 2000). Microsomes were incubated with 25 μM DCF-DA, 1 mM NADPH and 0–1 μM nicotine or cotinine in 37°C water bath for 30 min. DCF fluorescence was detected in 502/529 nm.

Western Blotting.

CYP2E1 and CYP2A5 were detected by Western blotting analysis. The SDS-PAGE and chemiluminescence imaging was carried out as described before (Lu et al. 2005). β-actin was detected as a protein loading control. Anti-CYP2E1 and anti-CYP2A5 antibodies were gifts from Dr. Jerome Lasker (Hackensack Biomedical Research Institute, Hackensack, NJ, USA) and Dr. Risto Juvonen (Department of Pharmacology and Toxicology, University of Kuopio, Kuopio, Finland), respectively. Anti- β-actin was from Santa Cruz Biotechnology.

Biochemical assays.

Serum and liver TG contents were measured using TG assay reagent (Thermo Fisher, Cat#: TR22421). Serum ALT activity was measured using a MaxDiscovery Alanine Transaminase (ALT) Color Endpoint Assay Kit (Bioo Scientific, Cat#: 3460–08) and serum glycerol was measured using glycerol assay kit (BioAssay Systems, Cat#: EGLY-200). Serum and urine cotinine levels were measured using cotinine ELISA kit (Abnova, Cat#: KA0930). Glutathione levels in the liver homogenate was measured using glutathione assay kit (Cayman Chemical, Cat #: 703002). Liver thiobarbituric acid reactive substances (TBARS) was measured as described before (Lu et al., 2005).

Statistical analyses

Results are expressed as mean ± S.D. Statistical evaluation was carried out by using one-way analysis of variance with subsequent the Student-Newman-Keuls post hoc test. $P<0.05$ was considered as statistical significance.

RESULTS

Both nicotine and cotinine enhance alcoholic fatty liver in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice

In experimental ALD, female rodents are more sensitive to chronic ethanol feeding than males. For example, in NIAAA model (acute-on-chronic model), males were selected because high mortality was expected in female mice (Bertola et al., 2013). In most experiments we selected female mice in chronic model (Lu et al., 2008; Lu et al., 2010; Lu et al., 2011). In this study, we still selected female mice. In an initial study, nicotine hydrogen tartrate salt was mixed in the liquid ethanol diet at 15 mg/L (32.5 μM). Alcohol induction of CYP2A5, which was observed in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice,

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was not affected by nicotine (Fig. 1A). Serum TG was increased by nicotine plus alcohol in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 1B). However, nicotine just slightly exacerbated alcoholic fatty liver in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 1C). When the dose of nicotine was increased to 30 mg/L (65 μM), the nicotine enhancing effect on alcoholic fatty liver was more evident and was still observed in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice as evaluated by H&E staining and Oil Red O staining (Fig. 2A), suggesting that the nicotine enhancing effect is CYP2A5-dependent. Cotinine is a major CYP2A5-generated metabolite of nicotine. Cotinine was fed to mice to test whether nicotine exerts its effect via cotinine. About 80% of nicotine are metabolized to cotinine, therefore, cotinine was mixed in the liquid diet at 65×80%=52 μM (9 mg/L) to make a good comparison with nicotine. Like nicotine, cotinine also enhanced alcoholic fatty liver in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 2A). To confirm the pathological observation, liver content of TG was measured. Consistently, liver TG contents were increased by ethanol in both *cyp2a5*$^{+/+}$ mice and *cyp2a5*$^{-/-}$ mice, but nicotine and cotinine further increased liver TG only in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 2B).

Like in the initial study, the combination of ethanol and nicotine induced hypertriglyceridemia in *cyp2a5*$^{+/+}$ mice rather than in *cyp2a5*$^{-/-}$ mice, but cotinine plus ethanol did not increase serum TG (Fig. 3A). Previously we reported that nicotine can enhance alcoholic fatty liver but it didn't enhance alcohol-induced liver inflammation (Lu et al. 2013). Consistently, ethanol-induced serum ALT was comparable in *cyp2a5*$^{+/+}$ mice and *cyp2a5*$^{-/-}$ mice, but neither nicotine nor cotinine further increased ethanol-induced serum ALT in both *cyp2a5*$^{+/+}$ mice and *cyp2a5*$^{-/-}$ mice (Fig. 3B). Alcohol-induced adipose lipolysis contributes to alcohol-induced fat accumulation in liver (Horning et al. 1960; Poggi and Di Luzio 1964; Zhong et al. 2012). Indeed, serum glycerol, a marker of adipose lipolysis, was increased by ethanol (Fig. 3C). Nicotine can also induce lipolysis in *in vitro* cultured adipocytes (Liu et al. 2004), but nicotine didn't further increase serum glycerol (Fig. 3C), suggesting that the enhancing effects of nicotine on alcoholic fatty liver are not through enhancing adipose lipolysis.

The half-life for nicotine is only 6–7 min in mice (Matta et al. 2007). About 80% of nicotine is metabolized to cotinine, and cotinine has a long half-life (15–40 h) (Davis et al. 2009). We measured cotinine in urine and blood. As expected, after nicotine feeding, urine cotinine concentrations were lower in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice (Fig. 4A). Interestingly, after cotinine feeding, urine cotinine was also lower in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice (Fig. 4A). Compared to urine cotinine levels, blood cotinine levels were lower after a 6 h of fasting. When nicotine and cotinine were fed in control diet, blood cotinine levels were comparable in *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice; but when they were fed in ethanol diet, blood cotinine levels in *cyp2a5*$^{+/+}$ mice were lower than those in *cyp2a5*$^{-/-}$ mice (Fig. 4A), which might be due to alcohol induction of CYP2A5 (Fig. 4B). CYP2A5 was induced by ethanol feeding only in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice, and nicotine and cotinine had no effect on CYP2A5 induction by ethanol (Fig. 4B). As expected, CYP2E1 activity was increased by ethanol feeding in both *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice, which is consistent with our previous observation (Lu et al. 2012). Like CYP2A5, CYP2E1 activity was not affected by nicotine and cotinine, either (Fig. 4B).

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Nicotine and cotinine exacerbate ethanol-induced oxidative stress in *cyp2a5*$^{+/+}$ mice rather than in *cyp2a5*$^{-/-}$ mice

Nicotine metabolism by CYP2A6 can also produce ROS and induce oxidative stress (Benowitz 2009; Ande et al. 2012). We examined whether oxidative stress contributes to the enhancing effect of nicotine on alcoholic fatty liver. Liver contents of glutathione were decreased following ethanol feeding, but nicotine and cotinine did not further decrease liver glutathione, both in *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice (Fig. 5A). In contrast, TBARS, a marker of lipid peroxidation (LPO) and oxidative stress, was increased by ethanol feeding in both *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice; nicotine and cotinine further increased liver TBARS, which was observed in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 5B). IHC staining showed that MDA, another LPO marker, was mainly located around central veins (Fig. 5C). While no central vein area was positively stained in mice fed with the control diet, with or without nicotine or cotinine, about 50% central vein areas were positively stained in ethanol-fed *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice, when ethanol was combined with nicotine or cotinine, about 90% positive staining in *cyp2a5*$^{+/+}$ mice but still about 50% in *cyp2a5*$^{-/-}$ mice (Fig. 5D). Similarly, after ethanol feeding, the 3-NT, a marker of nitrosative stress, was enhanced by nicotine and cotinine in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 5E, 5F). These results suggest that nicotine and cotinine enhance alcoholic fatty liver probably through exacerbating oxidative/nitrosative stress.

To directly detect ROS production, conversion of DCF-DA to DCF during nicotine and cotinine metabolism in microsomal systems was evaluated. When being incubated with nicotine or cotinine, microsomes from *cyp2a5*$^{+/+}$ mice caused a 4-fold elevation of fluorescent DCF production, but microsomes from *cyp2a5*$^{-/-}$ mice did not produce DCF (Fig. 6). These results suggest that CYP2A5 is essential for ROS production in microsomal metabolism of nicotine and cotinine.

DISCUSSION

Previously we reported that nicotine can enhance alcoholic fatty liver (Lu et al. 2013). Here we focused on the effect of CYP2A5 on nicotine-enhanced alcoholic fatty liver. We once reported that *cyp2a5*$^{-/-}$ mice developed more severe alcoholic fatty liver than *cyp2a5*$^{+/+}$ mice did (Hong et al. 2015; Chen et al. 2017). In this study, we modified animal feeding protocol to make alcoholic fatty liver comparable between *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice, thus it is easier to judge the difference in the nicotine enhancing effect on alcoholic fatty liver between *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice. Here we found that the nicotine enhancing effect on alcoholic fatty liver was observed in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice, suggesting that CYP2A5-dependent nicotine metabolism is indispensable for the nicotine enhancing effect on alcoholic fatty liver.

Nicotine is being used as a surrogate of tobacco cigarettes such as E-cigar, nicotine gum, nicotine patch, etc. Thus, respiratory system is not an only exposure route for nicotine. Dr. Xinxin Ding' group first evaluated nicotine metabolism in the *cyp2a5*$^{-/-}$ mice (Zhou et al. 2010). After nicotine injections, serum nicotine is much higher in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice; in contrast, serum cotinine is much lower in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice, confirming that CYP2A5 is the major nicotine oxidase (Zhou et al. 2010).

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However, in this study, when nicotine was fed in control diet, no significant difference in serum cotinine was observed between *cyp2a5*$^{-/-}$ mice and *cyp2a5*$^{+/+}$ mice; but when nicotine was fed in ethanol diet, serum cotinine was even higher in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice. The differences between us and Zhou et al are as follows: 1. In our model, nicotine was fed in liquid diet instead of being injected; 2. In our model nicotine was continuously administrated for 18 days, but Zhou et al collected blood within a few hours after one single nicotine injection. Actually, after injection of nicotine, a higher serum cotinine in *cyp2a5*$^{+/+}$ mice than in *cyp2a5*$^{-/-}$ mice was observed only within 1 hour; in contrast, a lower serum cotinine in *cyp2a5*$^{+/+}$ mice than in *cyp2a5*$^{-/-}$ mice was observed after 4 h and 8 h (Zhou et al. 2010). Nicotine might also be metabolized by other cytochrome P450s (Yue et al. 2009), which can explain why cotinine was still accumulated in *cyp2a5*$^{-/-}$ mice but can't explain why serum cotinine was even higher *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice. In our model, urine cotinine was lower in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice, thus, higher serum cotinine in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice might be due to lower excretion of cotinine via urine in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice. CYP2A5 is also expressed in kidney and cotinine is also metabolized by CYP2A5 (Su et al. 1998; Zhou et al. 2010). It will be interesting to address whether CYP2A5 in kidney will affect cotinine excretion in urine.

The half-life for plasma nicotine is only 6–7 min in mice, and cotinine is a major nicotine metabolite (Matta et al. 2007). Compared with nicotine, cotinine has a long half-life in plasma (6–7 min vs 15–40 h) (Davis et al. 2009). Nicotine exerts its cellular functions through nicotinic acetylcholine receptors (nAChRs) (Lindstrom 1997; Itier and Bertrand 2001). While nAChRs are widespread in neurons and neuromuscular junctions, nAChRs are also detected in non-neuronal cells such as adipocytes and nicotine can induce lipolysis and release of free fatty acids (FFA) in *in vitro* cultured adipocytes (Liu et al. 2004). But cotinine has lower affinity to nAchR than nicotine by over two orders of magnitude (Petri et al. 2001). Indeed, in the absence of ethanol, nicotine but not cotinine can induce adipose lipolysis (increases in serum glycerol, Fig. 3D). Alcohol-induced adipose lipolysis contributes to alcohol-induced fat accumulation in liver (Horning et al. 1960; Poggi and Di Luzio 1964; Zhong et al. 2012). However, nicotine did not enhance ethanol-induced adipose lipolysis. Therefore, nicotine enhancing effect on alcoholic fatty liver is not through enhancing adipose lipolysis. After ethanol plus nicotine feeding, level of serum cotinine was slightly higher in *cyp2a5*$^{-/-}$ mice than in *cyp2a5*$^{+/+}$ mice, but an enhancing effect on alcoholic fatty liver was still not observed in *cyp2a5*$^{-/-}$ mice. What is more, when cotinine instead of nicotine was mixed in the ethanol diet, an enhancing effect on alcoholic fatty liver was not observed in *cyp2a5*$^{-/-}$ mice while the enhancing effect was observed in *cyp2a5*$^{+/+}$ mice. All these suggest that nicotine enhances alcoholic fatty liver not through its metabolite cotinine.

There is an increase in lipid peroxidation in tobacco smokers (Marrow et al. 1995). Nicotine metabolism by CYP2A6 in cell culture can produce ROS and induce oxidative stress (Benowitz 2009; Ande et al. 2012). Nicotine might also be metabolized by other cytochrome P450s (Yue et al. 2009). We once reported that only being metabolized by CYP2E1 can a long-term administration of thioacetamide (TAA) induce oxidative stress and liver fibrosis (Hong et al. 2016). Similarly, only being metabolized by CYP2A5, nicotine may produce

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significant amount of ROS (Fig. 6). Cotinine can also be metabolized by CYP2A5 (Zhou et al. 2010). Like nicotine, cotinine also increased ROS generation. CYP2E1-generated ROS contributes to alcoholic fatty liver (Lu and Cederbaum 2008). CYP2E1 and CYP2A5 are co-located around central veins in liver (Lu et al. 2010). It will be challenging to distinguish the ROS induced by ethanol alone from those generated during the process of nicotine metabolism by CYP2A5. However, during the process of nicotine and cotinine metabolism, CYP2A5-mediated ROS and CYP2E1-mediated ROS during alcohol consumption may synergize to enhance alcoholic fatty liver, which happens in *cyp2a5*$^{+/+}$ mice but not in *cyp2a5*$^{-/-}$ mice. Our observation is in agreement with a recent report that aggravated oxidative stress promotes obesity-induced hepatic steatosis (Minato et al, 2014).

Chronic alcohol consumption can induce CYP2E1, which can metabolize ethanol and produce ROS, so CYP2E1 promote alcoholic fatty liver (Lu et al., 2008). CYP2A5 can also be induced by ethanol and ethanol induction of CYP2A5 is CYP2E1-dependent (Lu et al., 2010; 2012), but CYP2A5 does not metabolize ethanol and CYP2A5 is protective to alcoholic fatty liver (Lu et al., 2012; Hong et al., 2015; Chen et al., 2017). However, in the presence of nicotine or cotinine, CYP2A5 metabolizes nicotine or cotinine and produces ROS. CYP2A5 is mainly located in endoplasmic reticulum (ER), so ROS produced in ER may attack protein and cause accumulation of unfolded and misfolded protein in the ER i.e. ER stress. In the ER of the liver, synthesized TG bind to apolipoprotein B (apoB) and then form matured very low density lipoprotein (VLDL) for secretion into blood (Fisher and Ginsberg, 2002). Under oxidative stress and ER stress, misfolded apoB may retain in the ER and cause fat accumulation in liver (Su et al., 2009; Pan et al., 2004). Thus, nicotine-induced oxidative stress and alcohol-induced oxidative stress may combine together and make the alcoholic fatty liver exacerbated. Nicotine alone did not induce fatty liver, it only enhanced ethanol-induced fatty liver during the process of CYP2A5-dependent nicotine metabolism. Male mice express higher CYP2A5 with elevated catalytic activity than female mice, probably in male mice more ROS is produced during nicotine metabolism, but male mice are insensitive to ethanol-induced fatty liver. When nicotine and ethanol are combined together, will fatty liver be exacerbated in male mice to a similar extent to female mice? It will be interesting to address the issue of sex difference in nicotine enhancing effect on alcoholic fatty liver.

In conclusion, nicotine can enhance alcoholic fatty liver, and this nicotine enhancing effect was CYP2A5-dependent. The nicotine enhancing effect on alcoholic fatty liver is not through its metabolite because cotinine, a major nicotine metabolite produced by CYP2A5, can also enhance alcoholic fatty liver in a CYP2A5-dependent manner. Instead, the contributing role of CYP2A5 might be through oxidative stress generated during the process of CYP2A5-dependent nicotine metabolism because alcohol-induced oxidative stress is promoted by nicotine and cotinine in a CYP2A5-dependent manner.

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Abbreviation:

3-NT	nitrotyrosine adducts
ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
ALDH	acetaldehyde dehydrogenase
ALT	alanine transaminase
COH	coumarin 7-hydroxylase
CYP2A5	cytochrome P450 2A5
***cyp2a5*$^{-/-}$ mice**	CYP2A5 knockout mice
***cyp2e1*$^{-/-}$ mice**	CYP2E1 knockout mice
DCF	2',7'-dichlorofluorescein
DCF-DA	2',7'-dichlorofluorescin diacetate
FFA	free fatty acid
KCL	potassium chloride
MDA	malondialdehyde
nAChRs	nicotinic acetylcholine receptors
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PNP	p-nitrophenol
ROS	reactive oxygen species
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloride acetic acid
TG	triglyceride
WT	wild type.

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Highlights

- Nicotine augments alcoholic fatty liver, which is CYP2A5-dependent
- Nicotine metabolite cotinine also enhances alcoholic fatty liver in a CYP2A5-dependent manner
- Metabolism of nicotine and cotinine by CYP2A5 produces ROS
- Nicotine and cotinine enhance alcoholic fatty liver via elevated oxidative stress

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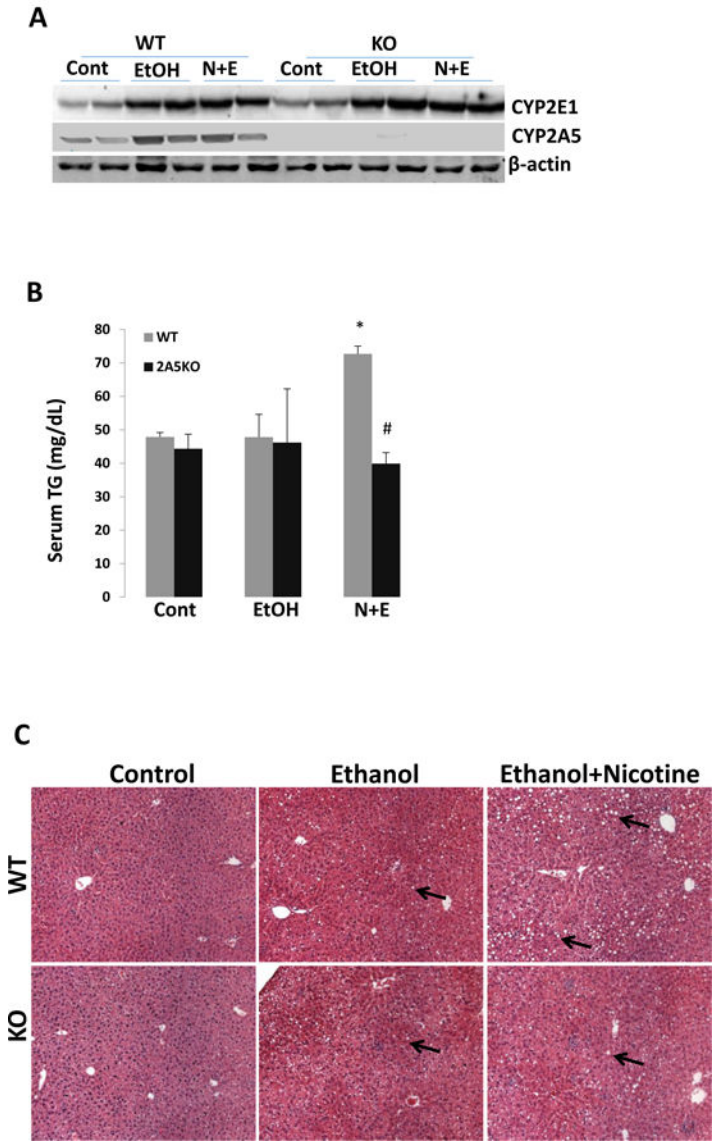

Figure 1.

Alcoholic fatty liver was enhanced by nicotine in cyp2a5$^{+/+}$ mice but not in cyp2a5$^{-/-}$ mice. The mice were fed liquid ethanol diet for 18 days, and nicotine was mixed in the ethanol diet at 15 mg/L in the form of hydrogen tartrate salt. **(A)** CYP2E1 and CYP2A5 induction by ethanol was not affected by nicotine. CYP2E1 and CYP2A5 were detected by Western blotting analysis. **(B)** Serum TG. **(C)** Liver sections with H&E staining. Arrows show lipid droplets. * P<0.05, compared with EtOH group; # P<0.05, compared with corresponding WT group. WT, cyp2a5$^{+/+}$ mice; 2A5KO, cyp2a5$^{-/-}$ mice; Cont, Control; N, nicotine; N+E, nicotine plus nicotine.

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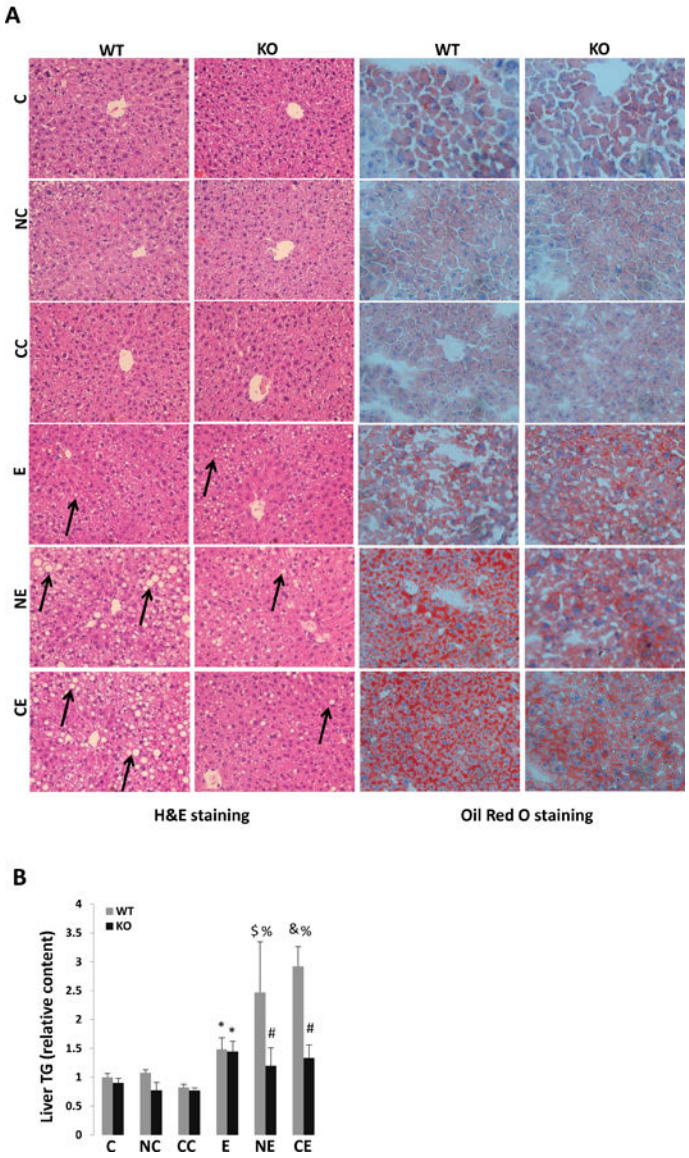

Figure 2.

Nicotine and cotinine enhance alcoholic fatty liver in cyp2a5$^{+/+}$ mice but not in cyp2a5$^{-/-}$ mice. The mice were fed liquid ethanol diet for 18 days; nicotine in the form of hydrogen tartrate salt was mixed in the ethanol diet at 30 mg/L (equivalent to 65 μM); cotinine was mixed in the liquid diet at 52 μM instead of 65 μM because nicotine was metabolized to cotinine at a rate of 80%. (A) H&E staining and Oil Red O staining. Arrows show macro-vesicular lipid droplets. (B) Liver TG content. *P<0.05, compared with C group; $ P<0.05, compared with NC group; & P<0.05, compared with CC group; # P<0.05, compared to corresponding WT Group; % P<0.05, compared with E group. WT, cyp2a5$^{+/+}$ mice; KO, cyp2a5$^{-/-}$ mice; C, control diet; NC, nicotine mixed in control diet; CC, cotinine mixed in control diet; E, ethanol diet; NE, nicotine mixed in ethanol diet; CE, cotinine mixed in ethanol diet.

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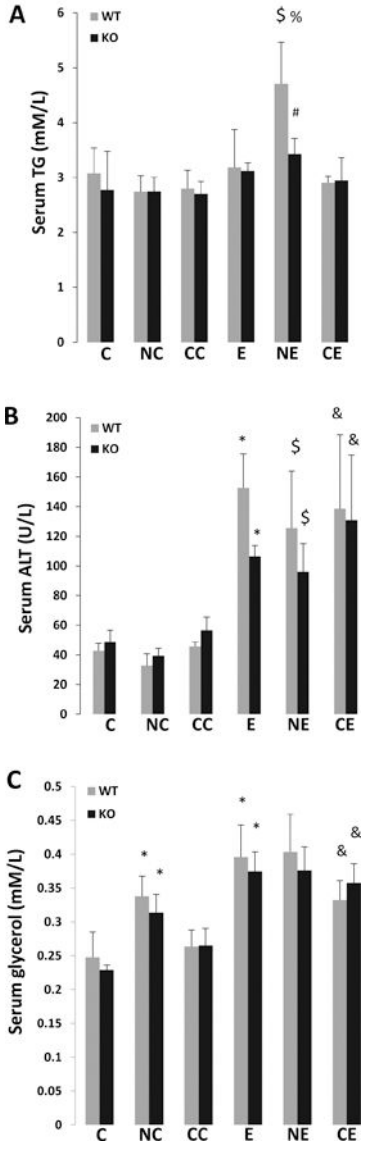

Figure 3.

Effects of nicotine and cotinine in combination with ethanol on serum TG (A), ALT (B), and glycerol (C). The mice were fed liquid ethanol diet for 18 days; nicotine in the form of hydrogen tartrate salt was mixed in the liquid diet at 65 μM; cotinine was mixed in the liquid diet at 52 μM. *P<0.05, compared with C group; $ P<0.05, compared with NC group; & P<0.05, compared with CC group; # P<0.05, compared to corresponding WT Group; % P<0.05, compared with E group. WT, cyp2a5$^{+/+}$ mice; KO, cyp2a5$^{-/-}$ mice; C, control diet; NC, nicotine mixed in control diet; CC, cotinine mixed in control diet; E, ethanol diet; NE, nicotine mixed in ethanol diet; CE, cotinine mixed in ethanol diet.

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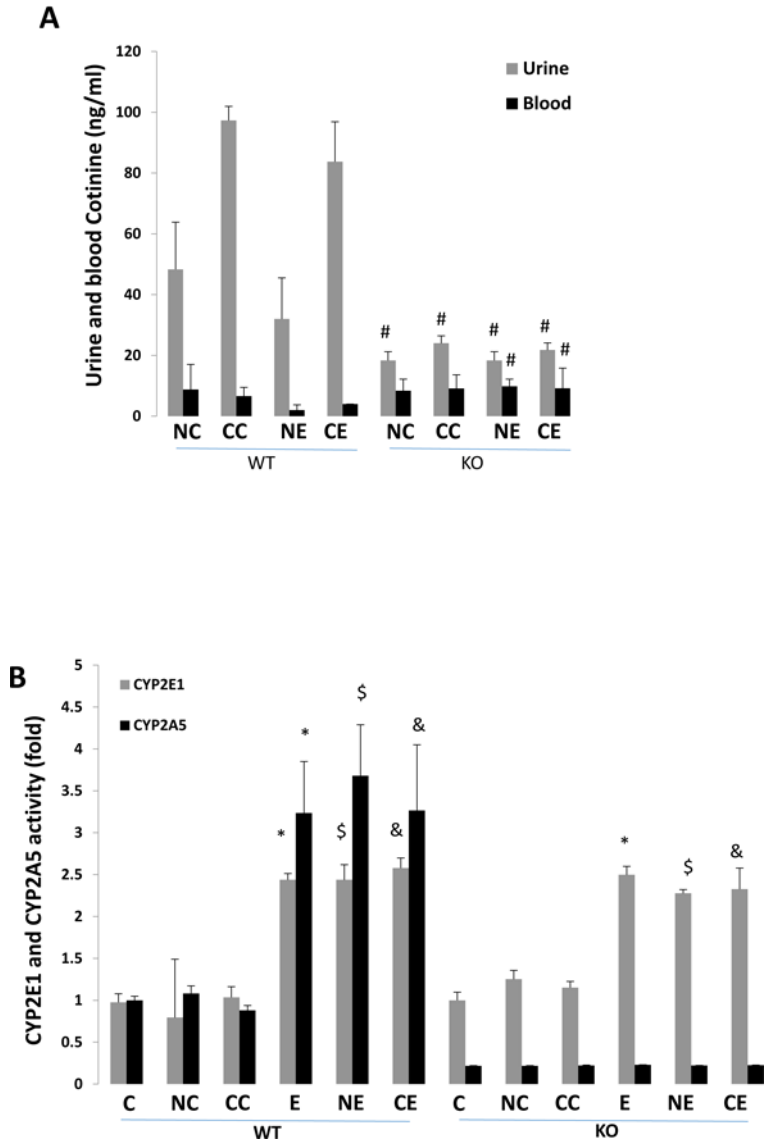

Figure 4.

Microsomal CYP2E1 and CYP2A5 activities (A) and cotinine concentration in urine and serum (B). Nicotine was mixed in the liquid diet at 65 μM and cotinine was mixed in the liquid diet at 52 μM. Urine was collected after 2 weeks of feeding, and blood was collected after being fed for 30 days. *P<0.05, compared with C group; $ P<0.05, compared with NC group; & P<0.05, compared with CC group; # P<0.05, compared to corresponding WT Group; % P<0.05, compared with E group. WT, cyp2a5$^{+/+}$ mice; KO, cyp2a5$^{-/-}$ mice; CC, control diet with cotinine; CE, ethanol diet with cotinine; NC, control diet wit nicotine; NE, ethanol diet with nicotine.

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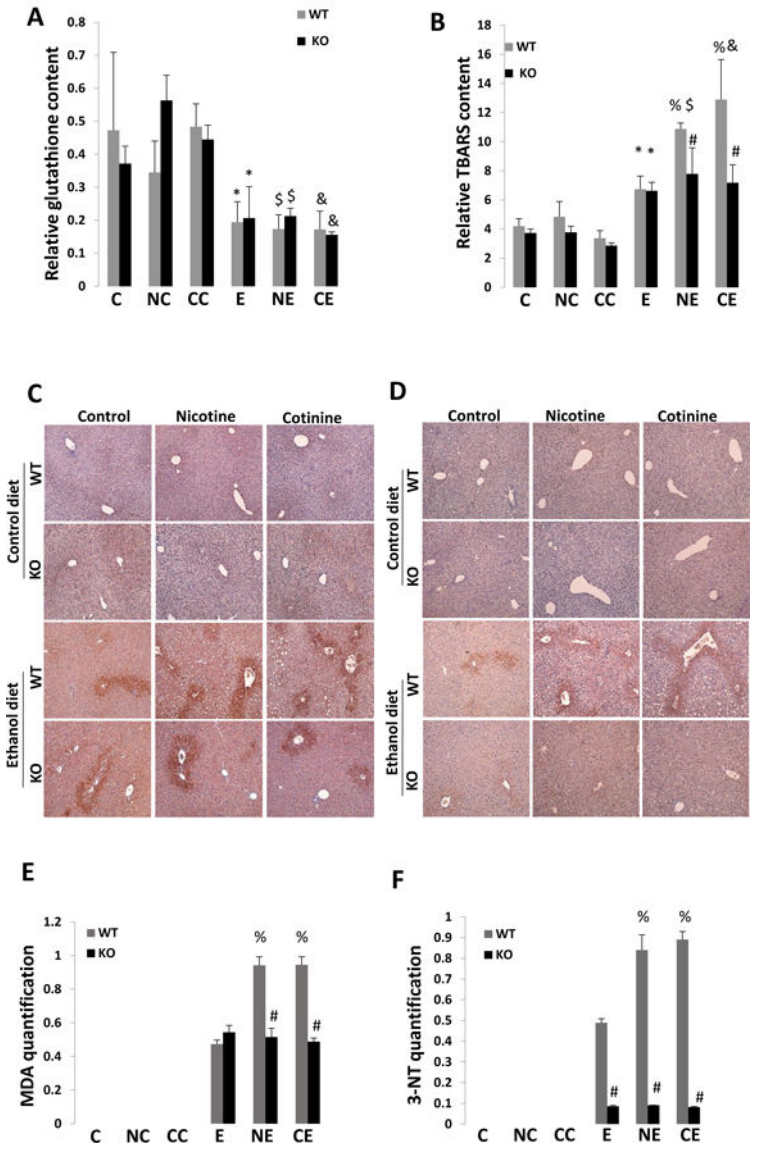

Figure 5.

Nicotine and cotinine enhance ethanol-induced oxidative stress. The mice were fed liquid ethanol diet for 18 days; nicotine and cotinine were mixed in the liquid diet at 65 μM and 52 μM, respectively. (A) Liver glutathione content; (B) Liver TBARS levels; (C) IHC staining for MDA in liver sections; (D) MDA quantification; (E) IHC staining for 3-NT in liver sections; (F) 3- NT quantification. $ $P<0.05$, compared with NC group; & $P<0.05$, compared with CC group; # $P<0.05$, compared to corresponding WT Group; % $P<0.05$, compared with E group.

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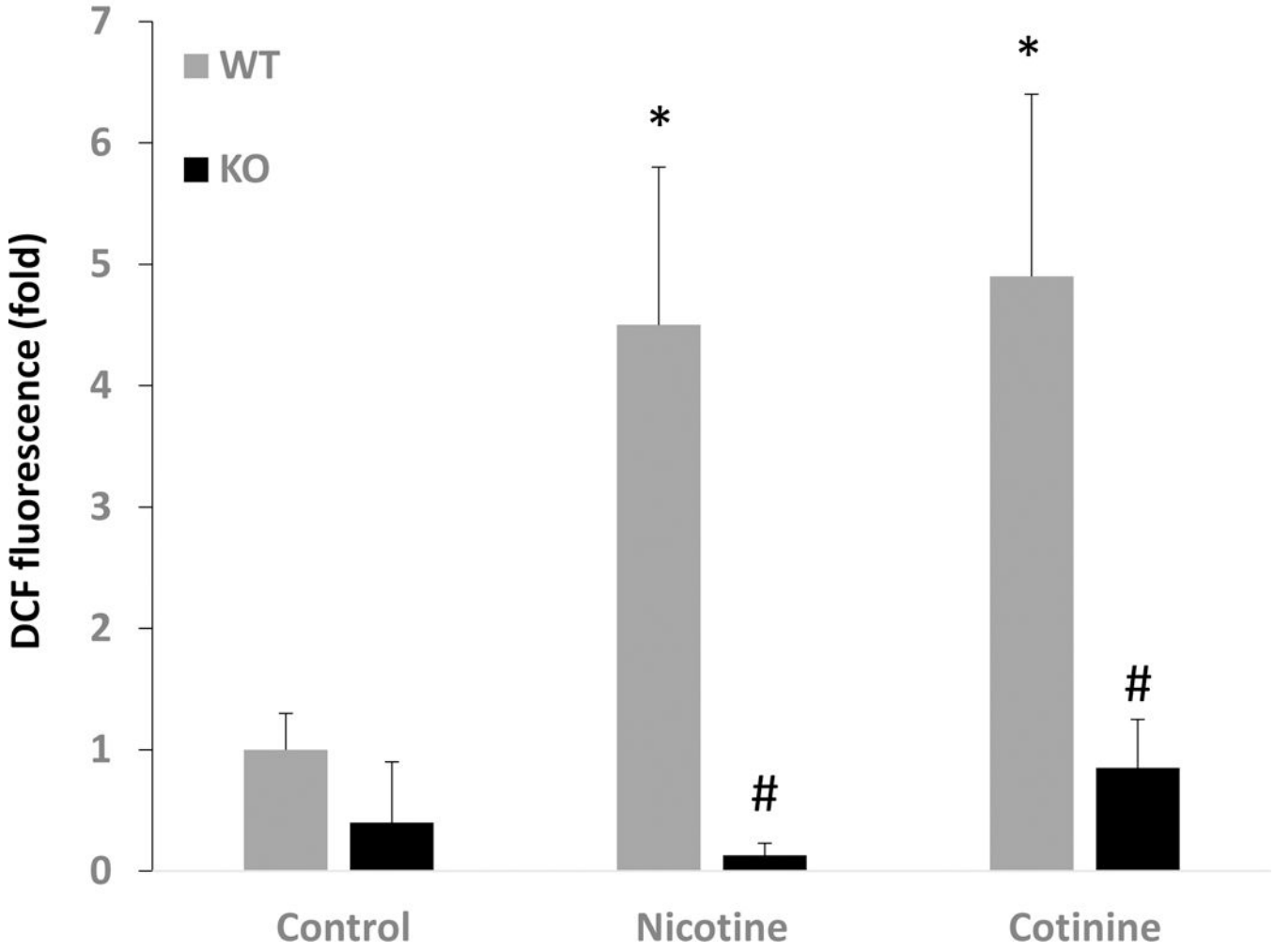

Figure 6.

Microsomal ROS production during nicotine and cotinine metabolism. Microsomes were isolated from livers of WT and KO mice, respectively. In the presence of 25μM DCF, 0–1 μM nicotine or cotinine were added to incubate in 37°C water bath for 30 min. Fluorescence was detected in 502/529 nm. *P<0.05, compared with Control group; # P<0.05, compared to corresponding WT Group.