Chronic exposure to e-cigarettes elevates CYP2A5 activity, protein expression, and cotinine-induced production of reactive oxygen species in mice

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ABSTRACT

Coumarin 7'-hydroxylase activity, a specific marker of CYP2A5 activity, and the protein level were measured in liver microsomes of male mice after chronic exposure to e-cigarettes (2.4% nicotine). After exposure for 240 min/day for 5 days, the activity and the protein level in preproenkephalin (ppENK) heterozygous (ppENK (+/-)) mice were significantly elevated ($p<0.05$) compared to the untreated control. This elevation was not due to deletion of the ppENK gene, because the activity did not differ among untreated ppENK (+/-), ppENK (-/-) and wildtype ppENK (+/+). Hence the elevation can reasonably be attributed to nicotine exposure. The production of reactive oxygen species (ROS) upon incubation of the hepatic microsomes of these mice with cotinine, was higher in microsomes from the e-cig-treated mice, compared to the untreated controls ($p<0.01$). Liquid-chromatography/mass-spectrometry assay showed three oxidation products of cotinine, viz trans 3'-hydroxycotinine (3'-HC), 5'-hydroxycotinine (5'-HC) and cotinine N-oxide (CNO) in the plasma of these mice. The result identifies these three oxidation reactions as the source of the observed ROS and also shows that, in nicotine-treated mice, the appropriate “nicotine metabolite ratio” is (3'-HC + 5'-HC + CNO)/cotinine. The results suggest intriguing possibilities that (i) this metabolite ratio may correlate with plasma nicotine clearance and hence impact nicotine’s psychoactive effects and (ii) chronic e-cig treatment causes ROS-induced oxidative stress which may play a major role in the regulation of CYP2A5 expression. Our present results clearly show that both the activity and the protein level of CYP2A5 are elevated by repeated exposure to nicotine.
SIGNIFICANCE STATEMENT

Nicotine, the psychoactive ingredient of tobacco, is eliminated as the oxidation products of cotinine in reactions catalyzed by the enzymes CYP2A5 in mice and CYP2A6 in humans. This study shows that repeated exposure to e-cigarettes elevates the level of CYP2A5 and the formation of reactive oxygen species. The results suggest an intriguing possibility that CYP2A5 may be up-regulated by chronic nicotine exposure due to oxidative stress caused by the oxidation of cotinine, in this preclinical model of human smokers.
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CYP2A5 elevation after chronic e-cigarette exposure in mice

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ABBREVIATIONS:
3'-HC, 3'-hydroxycotinine; 5'-HC, 5'-hydroxycotinine; CNO, cotinine N-oxide; COH, coumarin 7'-hydroxylase; COT, cotinine; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; LCMS, liquid-chromatography mass-spectrometry; MRM, multiple reaction monitoring; NMR, nicotine metabolite ratio; ppENK, preproenkephalin; ROS, reactive oxygen species.
INTRODUCTION

Nicotine, the psychoactive ingredient of e-cigarettes, is metabolized to cotinine by CYP2A6 in humans and by its close analogue, CYP2A5, in mice. Cotinine, the major biomarker of nicotine use, is oxidized to 3’-hydroxycotinine (3’-HC), a process that facilitates its renal elimination (Hukkanen et al., 2005). CYP2A5, present at a high level in the liver (Su et al., 1998), is induced by a variety of chemicals [reviewed by (Su and Ding, 2004)]. It is widely believed that the common factor among them is oxidative stress caused by the production of reactive oxygen species (ROS) (Abu-Bakar et al., 2013; Muhsain et al., 2015). The role of oxidative stress in regulating CYP2A5 is complex, and the mechanism is not fully understood. Furthermore, it is controversial whether repeated exposure to nicotine results in upregulation of CYP2A5 (Siu et al., 2006). Hence our first aim was to investigate the effect of chronic e-cig treatment on coumarin 7’-hydroxylase activity, a specific marker of CYP2A5 activity (Raunio et al., 2020) and on the protein level of CYP2A5 by Western blotting.

During our investigation on neurobehavioral effects of e-cig-exposure in ppENK (preproenkephalin)-heterozygous mice (see Discussion for details), we discovered that coumarin 7’-hydroxylase activity is significantly elevated in the liver microsomes of mice after exposure to e-cigarettes containing 2.4% nicotine for 240 min per day for 5 days (results section). We have used mice null in the ppENK gene, first characterized by (Konig et al., 1996), to examine the role of enkephalins in morphine tolerance (Marquez et al., 2006), alcohol reward (Tseng et al., 2013), and beiging (Brestoff et al., 2015). Because the elevated coumarin-7’-hydroxylase activity was observed in mice that were heterozygous in the ppENK gene, our second aim was to examine whether the
elevation was due to the deletion of this gene or to repeated exposure to nicotine, by measuring the activity in untreated ppENK (+/-), ppENK (-/-) and ppENK (+/+) mice. As CYP2A5 is known to be inducible by oxidative stress (Abu-Bakar et al., 2013), our third aim was to examine ROS production when liver microsomes from the chronically e-cig-treated male ppENK (+/-) mice were incubated with cotinine. The role of CYP2A5 in nicotine- and cotinine-induced ROS production was demonstrated by (Chen et al., 2018). They reported that upon incubation of liver microsomes from female C57BL/6 wildtype (CYP2A5 +/-) mice, ROS formation increased 4-fold in the presence of nicotine or cotinine, but ROS was not formed in CYP2A5 +/- mice. In male C57BL/6 mice, which have lower CYP2A5 activity than female mice (Kanamori et al., 2022), the rate of ROS production is unknown. Also, the impact of elevated microsomal CYP2A5 activity on ROS production has not been examined. Accordingly, we have addressed these issues in the present study.

Another unexplored avenue is the identity of cotinine oxidation pathways that are accompanied by ROS production in mice. The metabolic pathways of CYP2A5-catalyzed oxidation of nicotine to cotinine and of cotinine to its oxidation products are shown in Fig. 1. Plasma trans 3'-hydroxycotinine (trans 3'-HC) has been quantified after injection of cotinine (1 mg/kg)(Siu and Tyndale, 2007). However, 3'-HC has not been measured in the plasma of e-cig-treated mice, although a recent report of its presence in mouse urine after exposure to tobacco smoke (Sawa et al., 2021) provides indirect evidence for its formation at plasma nicotine levels resembling those of human smokers. Our recent study showed that, in addition to 3'-HC, 5'-hydroxycotinine (5'-HC) and cotinine N-oxide (CNO) are present in the plasma of wildtype male mice after e-cig
treatment for one day (Kanamori et al., 2022), but the effect of repeated e-cig treatment is unknown. Accordingly, our fourth aim is to quantify 3'-HC, 5'-HC and CNO in the plasma of chronically e-cigarette-treated ppENK (+/-) mice.

In humans, CYP2A6-mediated oxidation of cotinine to trans 3'-HC is well established (Hukkanen et al., 2005). The ratio of 3'-HC to cotinine, called nicotine metabolite ratio (NMR), measured in the plasma or saliva of smokers, is a well-established non-invasive marker of CYP2A6 activity (Benowitz, 2009). In humans, NMR was highly correlated with plasma nicotine clearance (Dempsey et al., 2004). In adolescent smokers, low NMR (i.e. slow nicotine clearance) was associated with an increased risk of acquiring nicotine dependence, probably due to prolonged exposure of the central nervous system to nicotine (El-Boraie and Tyndale, 2021). In mice, nicotine metabolite ratio has not been defined. Our fifth aim is to propose an appropriate nicotine metabolite ratio in mice based on cotinine oxidation products quantified in the present study.

MATERIALS AND METHODS

Chemicals and reagents

2',7'-Dichlorofluorescein (DCF), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Na₄NADPH, dimethyl sulfoxide (≥99.5%), (±)-nicotine-d₄ (0.1 mg/mL in acetonitrile), (±)-cotinine-d₃ (1 mg/mL in methanol), trans 3'-hydroxycotinine (1 mg/mL in methanol), trans 3'-hydroxycotinine-d₃ (0.1 mg/mL in methanol), coumarin, 7'-hydroxycoumarin and Bradford reagents were purchased from Millipore Sigma (St Louis, MI). (-)-Cotinine was purchased from Cayman Chemical (Ann Arbor, MI). 5'-hydroxycotinine (N-methyl 2-oxo...
3-pyridinebutanamide) was purchased from Santa Cruz Biotechnology (Dallas, Texas). (S)-cotinine N-oxide and (R,S)-cotinine-d₃ N-oxide were purchased from Toronto Research Chemicals (Toronto, Canada). Microsome isolation kit (BioVision K249-50) was purchased from Abcam (Milpitas, CA). SDS-polyacrylamide gel (#4561033) and the molecular weight standard (#1610373) were purchased from Bio-Rad Laboratories (Hercules, CA). Primary antibody to human CYP2A6 (ab3570), primary antibody to glyceraldehyde 3-phosphate dehydrogenase (ab9485), horse-radish peroxidase-conjugated secondary antibody (ab205718) and enhanced chemiluminescence substrate kit (ab 134406) were purchased from Abcam (Waltham, MA).

Animal treatment and preparation of plasma and liver

A total of 19 male mice (24–48 g) were used at the age of 3-8 months. Mice lacking the ppENK gene (n = 4), and their wildtype (n = 7) and heterozygous littermates/age-matched controls (n = 8) were bred in house. Mice were fully backcrossed on a C57BL/6J mouse strain, and were maintained on a 12 h light /12 h dark cycle (light on at 6:00 a.m.). All experiments were carried out according to the NIH guidelines for the proper care and use of animals in research and approved by the Institutional Animal Care and Use Committee (R21/IACUC/009) at the Western University of Health Sciences (Pomona, CA, USA).

Four ppENK (+/-) mice were used for chronic e-cig treatment. Each mouse was brought to the laboratory and habituated to the room for 1 h. Mice were then exposed to an e-cig containing 2.4% nicotine for 4 h (from 10:00 to 14:00). The e-cig system, EcigAero manufactured by AutoMate Scientific, Inc. (Berkeley, CA) described previously (Shao et al., 2019), was used with the following protocol in our laboratory at the
Western University of Health Sciences. It was programmed to generate e-cig aerosol exposure, i.e., ten puffs/h, with a puff duration of 4 sec and inter-puff interval of 10 sec, using Classic tobacco flavor bluCig PLUS e-cig tanks. This procedure was repeated for 5 days. The choice of the dose and duration of exposure was based on our earlier studies (Shao et al., 2019; Kanamori et al., 2022) which showed this treatment yields plasma levels of nicotine and cotinine in mice that are comparable to human smokers. On day 5, mice were sacrificed by cervical dislocation 5-6 min after the 4-h exposure period. The trunk blood was collected in 1.5-ml vials containing 50 μl of a 7% ethylenediamine tetra-acetic acid (EDTA) solution. The vials were then spun for 10 min at 14,000 rpm at 4 °C to collect the plasma samples. For the liver collection, the abdomen was carefully opened with a pair of sharp scissors and the liver was exposed and incised carefully. Plasma and liver tissues were stored at -80°C until analysis. Liver tissue was also collected from untreated (naïve) C57BL/6J wildtype mice (n = 7), ppENK (+/-) mice (n = 4) and ppENK (-/-) mice (n = 4), as described above.

Liver microsome preparation
Liver microsome was prepared from the frozen liver tissues with BioVision microsome isolation kit according to the manufacturer’s instruction, and as described recently (Kanamori et al., 2022). Briefly, the liver tissue was homogenized in the homogenizing buffer containing sucrose and protease inhibitor cocktail (1 mL/400 mg of tissue). The homogenate was centrifuged at 10,800 x g for 15 min to precipitate cell debris, and this procedure was repeated twice. The supernatant was then centrifuged at 38,700 x g for 20 min. This resulted in the separation of pink translucent microsomal
pellet. All operations were performed at 4 °C. The microsome pellet was suspended in the storage buffer and stored at -80 °C until use.

_Coumarin 7'-hydroxylase assay_

The activity was assayed as previously described (Aitio, 1978). Briefly, the assay mixture containing 100 μM coumarin, 1 mM NADPH and hepatic microsomes was incubated at 37 °C for 10 min. After terminating the reaction and precipitating microsomal protein, 7'-hydroxycoumarin in the supernatant was assayed by fluorometric detection at 360/460 nm (excitation/emission) in Biotek Synergy II microplate reader (Winooski, VT). The fluorescence in the assay mixture without incubation (control) was subtracted from the fluorescence in the incubated mixture, then converted to 7'-hydroxycoumarin concentration by calibration with the standard. Coumarin 7'-hydroxylase activity was calculated from the pmol of 7'-hydroxycoumarin formed/min/mg of microsomal protein. The reported activity is the mean of the activities determined at two different microsomal dilutions. Microsomal protein concentration was determined by Bradford assay.

_Western blotting_

Western blotting was performed as described previously (Siu et al., 2006)

To determine the linear range of CYP2A5 detection for the immunoblotting assay, mouse liver microsomes were serially diluted and used to construct standard curves (2.4 to 12.8 μg protein). To ensure accuracy in loading, the protein concentrations of the liver microsome from each of the four untreated and four e-cig-treated ppENK (+/-) mice were determined in duplicates by Bradford assay. Each microsomal sample was diluted with 50 mM Na₂HPO₄ buffer (pH7.4) to achieve a concentration of 6.4 μg
protein/10 μL, followed by the addition of 10 μL of the sample loading buffer and heating at 70 °C for 10 min for denaturation. After cooling, each sample was carefully loaded onto lanes 2 to 8 of the gel, separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide), and transferred to a nitrocellulose membrane by tank transfer at 100 V for 1 h 50 min. For the detection of CYP2A5, nitrocellulose membranes were preincubated for 1 h in a blocking solution containing 1% skim milk powder (w/v), and 0.1% bovine serum albumin (w/v) in Tris-buffered saline-Triton X-100 [20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (v/v) Triton X-100]. Membranes were probed with a primary antibody to human CYP2A6 (1:3,000 dilution) and a horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a house-keeping protein, was used as the loading control and probed with the primary antibody at 1:1000 dilution followed by incubation with the secondary antibody. The molecular weights of the detected CYP2A5 and GAPDH bands were confirmed by running molecular weight standards containing 50 kDa and 37 kDa standards in lanes 1 and 10. The protein bands of the liver microsomal samples were detected by enhanced chemiluminescence, and of the molecular weight standards by colorimetric assay on BioRad ChemiDoc XRS+ image system. The protein density was quantified by BioRad Image Lab software v.6.1.

**Cotinine-induced microsomal ROS production.**

ROS production was measured by 2′,7′-dichlorofluorescein (DCF) method by incubating the liver microsome with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Serron et al., 2000). The nonfluorescent probe DCFH–DA crosses the cell membrane (Bass et
and undergoes hydrolysis by endogenous esterase to nonfluorescent DCFH. DCFH is then rapidly oxidized in the presence of reactive oxygen species (ROS) to highly fluorescent DCF (Szejda et al., 1984; Lebel and Bondy, 1990). Hence, the production of DCF is proportional to ROS.

DCF standard was prepared as 1 mM stock solution in dimethyl sulfoxide and stored at -20 °C. The stock solution was serially diluted with 50 mM Na₂HPO₄ buffer (pH 7.4) to prepare DCF standards at concentrations of 100 to 100,000 nM and stored at -20 °C until use. DCFH-DA was prepared as stock solution in methanol (1.25 mM), then diluted to 250 μM with Na₂HPO₄ buffer (pH 7.4). Microsomal ROS production during cotinine metabolism was measured as described by (Chen et al., 2018), with modifications described below which were essential for successful assay in our laboratory. Cotinine (400 μM in Na₂HPO₄ buffer (pH7.4)), NADPH (10 mM in Na₂HPO₄ buffer (pH 7.4)) and DCFH-DA (250 μM) were prepared fresh just before use. Microsome was diluted to 0.05 mg/10 μL of Na₂HPO₄ buffer (pH 7.4), based on prior Bradford assay of microsomal protein concentration. The complete DCF assay mixture contained 25 μM DCF-DA, 1 mM NADPH, 40 μM cotinine, and 0.05 mg of microsome, prepared by addition of 10 μL of each and Na₂HPO₄ buffer (pH 7.4) to the final volume of 100 μL. The order of addition in successful assay was as follows. To the assay solution containing 25 μM DCF-DA, 0.05 mg of microsome was added and incubated for 15 min at 37 °C to allow uptake of the non-fluorescent probe DCF-DA into the microsome and its conversion to DCFH by endogenous deacetylase. Then NADPH (1 mM) was added to initiate oxidation reactions catalyzed by microsomal P450 enzymes. Finally, 40 μM cotinine was added to monitor cotinine-induced DCF production.
modification crucial for successful assays under our conditions was the increase of cotinine concentration to 40 μM. We selected this concentration because a previous study showed that production of 3'-HC from cotinine in vitro upon incubation with liver microsomes of C57BL/6 mice reaches near-maximum at cotinine concentration of 40 μM (Siu and Tyndale, 2007). Each assay mixture was prepared in at least four microplate wells to check reproducibility. Immediately after the addition of cotinine, the 96-well black microplate, containing DCF standards and the assay mixtures, with or without cotinine, was placed in Biotek Synergy II microplate reader (Winooski, VT) that had been preincubated to 37 °C. DCF formation was measured fluorometrically at the excitation and emission wavelengths of 485 and 528 nm respectively with reading (in duplicates) taken every 5 or 10 min for 1 h to measure the time-course of ROS-induced DCF production.

For data analysis, the fluorescence at $t = 0$ was subtracted from the fluorescence at $t$ for each data point, then divided by the microsomal protein concentration of 0.05 mg to obtain DCF formation in pmol/mg protein. Then the mean value of pmol/mg protein was calculated for $n = 4$ data points in each group (control and with 40 μM cotinine). The time interval where DCF production is linear was determined and the rate of DCF formation in pmol/min/mg protein was calculated. The rate of formation in the absence of cotinine was subtracted from the rate in the presence of cotinine to obtain DCF formation due to cotinine oxidation in pmol/min/mg microsomal protein. Alternatively, the rate of cotinine-induced ROS production was also calculated by subtracting DCF formation in the absence of cotinine from that in the presence of cotinine at each time.
point $t$, then calculating the rate over the period of linear increase. The two methods provided virtually the same rate of cotinine-induced DCF production.

**LCMS assay of plasma nicotine and its metabolites**

LCMS experiments were performed on an Acquity UPLC system coupled to a Xevo ToF mass spectrometer (Waters Corporation, Milford, MA, USA), located in the Water and Environment Laboratory at California Institute of Technology, as described in detail previously (Kanamori et al., 2022). Nicotine was assayed by liquid-chromatography/tandem mass-spectrometry using multiple reaction monitoring (MRM) of its product ion with m/z 130.06 (Nguyen et al., 2020). Cotinine, 3’-HC, 5’-HC and CNO were monitored by direct high-resolution MS through the selection of the parent ion $[M+H]^+$ with m/z of 177.1062 for cotinine and $[M+H]^+$ with m/z of 193.0977 for 3’-HC, 5’-HC and CNO which share the same parent ion, but are resolved by different retention times (see results). Table 1 lists the retention times, m/z of the parent ion $[M+H]^+$ of each standard, its deuterated isotopomers, and the multiple reaction monitoring parameters for nicotine.

Nicotine, cotinine, 3’-HC, 5’-HC and CNO standards in the plasma matrix were prepared in the same way as the plasma samples, as described in detail previously (Kanamori et al., 2022). Calibration graphs of the standards, used to quantify plasma metabolites, are shown in Supplemental data Fig. S1. Precision, defined as the closeness of measured values at the same concentration, was assessed by duplicate LCMS assays of each plasma sample, and calculating the percentage difference between the two assays.

*Calculation of nicotine metabolite ratio.*
The nicotine metabolite ratio was calculated as (3'-HC + 5'-HC + CNO)/cotinine in ng/mL, then converted to molar ratio by multiplication with 176.2/192.2 to correct for the different molecular weights of cotinine vs its three oxidation products.

**Statistical Analysis**

Data are expressed as the mean ± standard deviation. Data were analyzed by unpaired parametric two-tailed t-test, Mann-Whitney U test, or one-way ANOVA, whichever is appropriate as indicated in each figure legend, using GraphPad Prism 9 (GraphPad Software, Inc. San Diego, CA).

**RESULTS**

**Coumarin 7'-hydroxylase activity in chronic e-cig-treated mice**

Fig. 2 shows coumarin 7'-hydroxylase activity, as a specific marker of CYP2A5 activity, in the liver microsomes of male ppENK (+/-) mice (n = 4) after chronic e-cig treatment. The mice were exposed to e-cigarettes containing 2.4% nicotine for 240 min/day for 5 days, with the liver collected on day 5. The median in the treated mice, 89.35 ng/mL, is higher than that in the untreated, 23.85 mg/mL, with p = 0.0286 by Mann-Whitney U test. The result shows that chronic e-cig treatment elevates coumarin 7'-hydroxylase activity in male ppENK (+/-) mice.

To examine whether these changes are due to e-cig exposure or the missing of an enkephalin allele, we used naïve mice lacking the ppENK gene and their wildtype and heterozygous littermates/controls. Fig. 3 compares coumarin 7'-hydroxylase activities, as the mean ± SD, in the liver microsomes of untreated male ppENK (+/-) (n = 4), ppENK (-/-) (n = 4) and wildtype mice (n = 7). One-way ANOVA revealed no
significant difference in the activities of this enzyme among these genotypes ($p = 0.54$).

Taken together, the results show that the elevation of CYP2A5 in the microsomes of ppENK (+/-) mice after chronic e-cig-treatment seen in Fig. 2 is due to repeated exposure to nicotine, and not due to deletion of the ppENK gene.

*Western blot of CYP2A5 protein*

Fig. 4A shows a representative immunoblot of liver microsomal CYP2A5 at various dilutions of the microsomal protein. CYP2A5 protein is selectively observed at 50 kDa after incubation with the primary and secondary antibodies and chemiluminescent detection (Materials and Methods). Fig. 4B plots CYP2A5 density as the mean ± SD of three independent assays; the density increases linearly with protein over 2.4 to 12.8 µg. Fig. 4C (the upper image) shows a representative immunoblot of liver microsomal CYP2A5 from each of the four untreated ppENK(+/-) mice (the left four lanes) and from the e-cig-treated ppENK(+/-) mice (the right four lanes) when 6.4 µg of microsomal protein was loaded in each lane. The CYP2A5 protein density is higher in the four e-cig-treated mice than in the four untreated mice. Because the microsomal protein in each animal was measured in duplicates and the mean value used, and care was taken to load exactly 6.4 µg of the protein (Materials and Methods), we expect loading error to be minimal. However, for verification, we examined the protein expression of GAPDH, a house-keeping protein with a molecular weight of 37.5 kDa as the loading control, as shown in the lower image of Fig. 4C. The GAPDH densities in the four treated mice are lower than those in three of the four untreated ones as seen in the image and shown quantitatively in Supplemental data Table S1; this result shows that the higher CYP2A5 densities observed in the four treated mice are not due to differences in loading, but
represent higher levels of the CYP2A5 protein in the treated compared to the untreated. Fig. 4D compares the CYP2A5 densities of the four untreated ppENK-HET mice with those of the four e-cig-treated ppENK-HET mice, shown as the dot plot for each mouse. Each data point is the mean of three independent immunoblots from each mouse. The mean CYP2A5 density of the e-cig-treated ppENK-HET mice (shown by the horizontal line) is significantly higher than that of the untreated mice with \( p = 0.015 \) by unpaired parametric two-tailed \( t \)-test (\( t=3.39, \text{df}=6 \)). The result shows that repeated nicotine treatment via e-cig exposure elevates the protein level of liver microsomal CYP2A5.

*Cotinine-induced ROS production*

Fig. 5A (upper panel) shows the progressive production of DCF, as a measure of ROS, when the liver microsome of a male ppENK (+/-) mouse with chronic e-cig treatment was incubated without or with 40 \( \mu \)M cotinine. The rate of DCF formation was linear during \( t = 20-60 \) min and was faster in the presence of cotinine than in its absence (control) \( (n = 4 \) assays per data point, \( p < 0.01 \) at \( t = 20-30 \) min, \( p < 0.05 \) at \( t =35-60 \) min by unpaired \( t \) test). DCF production in the absence of cotinine arises from oxidation reactions of endogenous substrates catalyzed by microsomal P450 enzymes other than CYP2A5. The rate of DCF formation due to cotinine oxidation, calculated as described in Materials and Methods, was 21.9 pmol/min/mg microsomal protein in this mouse. By contrast, in the microsome from an untreated ppENK (+/-) mouse, the rate of DCF production due to cotinine oxidation was 3.2 pmol/min/mg protein (Fig. 5A lower panel). Fig. 5B shows the rate of cotinine-induced DCF formation in the microsomes of chronic e-cig-treated ppENK (+/-) mice compared with that in untreated ppENK (+/-) mice, as the mean \( \pm \) SD of 4 animals/treatment. The mean rate in the treated mice was higher.
than that in the untreated mice with \( p = 0.0086 \) by unpaired parametric two-tailed \( t \)-test \((t = 3.84, \text{df}=6)\).

**Nicotine metabolites in chronic e-cig-treated mice by LCMS**

Fig. 6 shows representative LCMS chromatograms of nicotine, cotinine, 3'-HC, 5'-HC and CNO in the plasma samples of a male ppENK (+/-) mouse which had undergone chronic e-cig treatment. The plasma was collected on day 5, 5-6 min after the last exposure. Plasma nicotine (Fig. 6A) and cotinine (B) are clearly observed. Furthermore, the three oxidation products of cotinine, viz 3'-HC (C), 5'-HC (D) and CNO (E), which had not been reported previously in the plasma of e-cig-treated mice (Introduction), are clearly detected. As shown in Fig. 6D, 5'-HC is well resolved from, and as abundant as, 3'-HC. The peak area of CNO is much smaller than the areas of 3'-HC and 5'-HC, because the CNO signal is suppressed due to its high polarity (Fig. 1) (Kanamori et al., 2022). Nevertheless, plasma CNO is measurable, as shown in the inset to Fig. 6E. The observed peak area, or the response = peak area*IS conc./IS area, where IS is the corresponding deuterated internal standard, was used to calculate the metabolite concentration in each plasma by comparison with the calibration graphs of the respective standards which are provided in Supporting information (Fig. S1).

Fig 7A shows the plasma nicotine, cotinine, 3'-HC, 5'-HC and CNO concentrations, as the mean ± SD of \( n = 4 \) in the ppENK (+/-) mice after chronic exposure to e-cig containing 2.4% nicotine. The precision of our concentration measurements is demonstrated by the fact that the percentage differences between the 1\textsuperscript{st} and 2\textsuperscript{nd} LCMS assay of plasma metabolite concentrations, were \(< 5.5\% \) for 3'-HC and \(<1.8\% \) for 5'-HC. In the nicotine-exposed mice (Fig. 7A), the plasma concentrations
of 3'-HC and 5'-HC are nearly as high as that of cotinine. The abundance of 3'-HC and 5'-HC in the plasma of these mice strongly suggests that the oxidation of cotinine to these products, catalyzed by microsomal CYP2A5, accounts for the observed cotinine-induced ROS production in Fig. 5.

Another notable feature is that the substrate cotinine shows little inter-animal variability; the coefficient of variation, viz (the standard deviation/mean) x 100 % is 13.3%. By contrast, there is high inter-animal variability in the oxidation products, 3'-HC, 5'-HC, and CNO and consequently in the combined concentration of (3'-HC + 5'-HC + CNO). The combined concentration has the mean ± SD of 156.8 ± 66.59 ng/mL, and hence a coefficient of variation of 42%. To examine whether this inter-animal variability reflects variability in coumarin 7'-hydroxylase activity (Fig. 2), we divided the plasma (3'-HC + 5'-HC + CNO) concentration in each mouse by its microsomal coumarin 7'-hydroxylase (COH) activity, i.e. [plasma (3'-HC + 5'-HC + CNO) concentration]/COH activity. Then we calculated the mean ± SD value of this ratio which, as shown in Fig. 7B, was 1.61 ± 0.29, with a coefficient of variation of 18 %. Thus, the coefficient of variation is much reduced, from 42% to 18%, when we examine the plasma (3'-HC + 5'-HC + CNO) concentration normalized to the COH activity. As stated earlier, the substrate cotinine has a low coefficient of variation, so its level is unlikely to cause much inter-animal variability in the rates of 3'-HC, 5'-HC and CNO production. Taken together, it is reasonable to conclude that the major factor that controls the plasma levels of 3'-HC, 5'-HC and CNO in this group of chronically e-cig-exposed mice is the coumarin 7'-hydroxylase activity in each animal.

Nicotine metabolite ratio in mice
The abundance of 5′-HC and the measurable level of CNO in Fig. 7A show that the appropriate “nicotine metabolite ratio” in the plasma of nicotine-treated mice is \((3′-\text{HC} + 5′-\text{HC} + \text{CNO})/\text{cotinine}\). Fig. 7C plots \((3′-\text{HC} + 5′-\text{HC} + \text{CNO})/\text{cotinine}\) in molar ratio (Materials and Methods) in each mouse vs. its coumarin 7′-hydroxylase activity to examine possible correlation. Although the number of animals is small, the correlation coefficient \((R=0.897)\) is high. The coumarin 7′-hydroxylase activity at the \(y\)-intercept, 34.7 pmol/min/mg, represents the extrapolated activity when the plasma \((3′-\text{HC} + 5′-\text{HC} + \text{CNO})\) concentration is zero, which corresponds to mice unexposed to nicotine with a constitutive level of CYP2A5. This extrapolated activity is very close to the median coumarin 7′-hydroxylase activity in untreated male WT C57BL/6 mice \((n = 8)\) which was 34.4 pmol/min/mg as reported in our recent study (Kanamori et al., 2022). Taken together, the results show that the proposed nicotine metabolite ratio for mice, \((3′-\text{HC} + 5′-\text{HC} + \text{CNO})/\text{Cot}\) is highly correlated with hepatic microsomal CYP2A5 activity. The mean ± SD of the nicotine metabolite ratio/COH activity was \(0.01826 ± 0.0048 \text{ min}·\text{mg·pmol}^{-1}\) \((n = 4)\) (not shown in the figure). The implications of the results are described below.

DISCUSSION

Previous studies have shown that nicotine causes alterations in the level of enkephalin and its precursor mRNA in the central nervous system and peripheral tissues (Van Loon et al., 1991; Dhatt et al., 1995). However, Ugur and colleagues did not find changes in proenkephalin mRNA although they observed changes in the level of delta opioid receptors in brain areas involved in reward and addiction (Ugur et al., 2017).
Conversely, enkephalins play a functional role in nicotine-induced antinociception, reward and physical dependence (Berrendero et al., 2005). Acute and chronic nicotine treatment have also been shown to alter the level of enkephalins in the periphery (Van Loon et al., 1991), suggesting that enkephalin may play a role in the peripheral actions of nicotine such as stress regulation, energy homeostasis and possibly its metabolism in the liver. In the present study, we sought to assess if repeated nicotine exposure via e-cig would alter the expression and activity of nicotine metabolizing enzyme, CYP2A5, in ppENK heterozygous mice.

Microsomal CYP2A5 activity was elevated after chronic e-cigarette exposure in male mice heterozygous in ppENK gene (Fig. 2). The elevation was not due to deletion of the ppENK gene (gene ID1861900 located on chromosome 4) because the activity of its microsomal CYP2A5 (gene ID13087 located on chromosome 7) was not different among untreated ppENK (+/-), and their ppENK (-/-) and wildtype littermates (Fig. 3). The protein level of CYP2A5, measured by Western blot, was higher in the four e-cigarette-treated ppENK (+/-) mice compared to the four untreated controls with p<0.05 (Fig. 4D). Taken together, the results clearly show that the activity and the expression of CYP2A5 were elevated as a result of repeated exposure to e-cigarette (2.4% nicotine; 240 min/day for 5 days). By contrast, in male wildtype mice, exposed to e-cigarettes with the same nicotine dose for only one day (n = 3), the microsomal CYP2A5 activity did not differ from that in untreated wildtype male mice (Kanamori et al., 2022).

The rate of ROS production, as measured by DCF production, was significantly higher when the microsomes from ppENK (+/-) male mice with chronic e-cig treatment was incubated with cotinine compared to that in untreated ppENK (+/-) mice (Fig. 5A
and B). The result clearly shows that chronic e-cig treatment enhances cotinine-induced ROS production in vitro. A previous study measured ROS production upon incubation of hepatic microsomes from female C57BL/6 mice, not only with cotinine, but also with nicotine (Chen et al., 2018). They reported that, with either nicotine or cotinine as the substrate, ROS production was four-fold higher in microsomes from CYP2A5<sup>+/+</sup> wild type mice compared to those from CYP2A5<sup>−/−</sup> knockout mice; their result demonstrated the importance of CYP2A5 in ROS production. The novel aspects of our work are the following. First, we report, for the first time, the time-course of cotinine-induced ROS formation (Fig. 5A) and a detailed protocol for this assay. Secondly, we provide the first quantitative comparison of the rates of cotinine-induced microsomal ROS production (in pmol/min/mg) in chronically e-cig-treated male mice vs. the untreated counterparts (Fig. 5B). Thirdly, we found that cotinine-induced ROS production is substantial even in male mice which have lower CYP2A5 activity than female mice when unexposed to nicotine (Kanamori et al., 2022).

We measured ROS production only with cotinine for the following reasons. Nicotine is oxidized by CYP2A5 to nicotine-Δ1'-Δ5'-iminium ion (Fig. 1). Conversion of the nicotine iminium ion to cotinine requires a second enzyme, cytosolic aldehyde oxidase, which is absent in microsomal preparations used by us and other laboratories (Siu and Tyndale, 2007; Zhou et al., 2010). Without an added source of the aldehyde oxidase, nicotine-induced ROS production is expected to result from the formation of the nicotine iminium ion alone and does not include those produced by the downstream oxidation of cotinine. A preliminary assay in our laboratory showed that DCF formation with added nicotine (100 µM in the assay solution) was lower than that with added
cotinine (40 μM) in the absence of cytosolic aldehyde oxidase. Our aim was to examine ROS formation resulting from cotinine oxidation. Accordingly, we did not examine ROS production in the presence of nicotine.

To identify cotinine oxidation products that are associated with the observed ROS production, we used LCMS to measure nicotine, cotinine and their metabolites in the plasma of these mice. Our results in Fig. 7A showed that after chronic e-cig treatment, male ppENK (+/-) mice have a plasma nicotine level of 73.8 ± 28.8 ng/mL and plasma cotinine level of 82.0 ± 10.9 ng/mL. These levels are in good agreement with those reported in human e-cig users (Hukkanen et al., 2005; Flouris et al., 2013). They are also in reasonable agreement with those measured in C57BL/6 mice exposed to tobacco smoke (Kaisar et al., 2018) and with those measured in e-cig treated Apolipoprotein E null (ApoE/-) male mice on a C7Bl/6J background, using nicotine doses and exposure times (Shao et al., 2019) that are different from ours.

Our results in Fig. 6 and Fig. 7A also demonstrate the feasibility of measuring the oxidation products of cotinine, namely 3’-HC, 5’-HC and CNO which, to the best of our knowledge, had not been reported previously in the plasma of e-cig-treated mice. Our finding that 5’-HC is nearly as abundant as 3’-HC strongly suggests that the ROS observed in Fig. 5 was released during oxidation of cotinine to 3’-HC, 5’-HC and, to a lesser extent, CNO.

Our results show that, for mice, the appropriate nicotine metabolite ratio is (3’-HC + 5’-HC + CNO)/cotinine. 3’-HC, 5’-HC and CNO are formed from cotinine by a single enzyme CYP2A5 (Fig. 1) and plasma cotinine has a relatively long half-life of 38 min (Siu and Tyndale, 2007; Zhou et al., 2010). Thus, this proposed nicotine metabolite
ratio for mice meets some of the criteria for ideal metabolic ratio described by (Dempsey et al., 2004) for the human 3'-HC/cotinine ratio, although, for mice, the stability of the NMR ratio over time and its correlation with plasma nicotine clearance remain to be examined. In human studies, NMR is useful as a non-invasive marker of CYP2A6 activity and of plasma nicotine clearance (Introduction). In mice, CYP2A5 activity is measurable in hepatic microsomes. Nevertheless, NMR, if established as a biomarker of nicotine clearance, can be valuable in neurobehavioral studies of chronically e-cig-treated mice. It will permit monitoring the temporal changes in nicotine clearance (and hence its psychoactive effect) with little physiological perturbation, for example, by tail vein blood sampling, or non-invasively by saliva collection, as has been reported in human smokers (Dempsey et al., 2004; Lea et al., 2006) (St Helen et al., 2012).

To the best of our knowledge, elevation of hepatic CYP2A6 and/or pulmonary CYP2A13 using NMR as their non-invasive biomarker, has not been reported in chronic users of e-cigarettes among humans. If elevation occurs, nicotine clearance is expected to increase, resulting in possible alleviation of its psychoactive effects. This is another intriguing area for future investigation.

Taken together, our results suggest an intriguing possibility that chronic e-cig treatment may cause ROS-induced oxidative stress in the liver which is thought to play a major role in the regulation of CYP2A5 (Abu-Bakar et al., 2013; Muhsain et al., 2015). A previous report (Siu et al., 2006) showed that CYP2A5 was higher in male high-nicotine-consuming C57Bl/6 mice, and induction of CYP2A5 as a result of nicotine consumption was considered to be a potential explanation of this finding. Our present
results show that both the activity and the protein level of CYP2A5 are elevated by repeated exposure to nicotine via e-cigarettes.

Production of reactive oxygen species (ROS) causes oxidative stress when it exceeds anti-oxidant defenses to eliminate them and the free radicals attack lipids, proteins and DNA (reviewed by (Arauz et al., 2016). E-cig exposure has been shown to cause DNA damage and mitochondrial dysfunction in mouse hepatocytes (Espinoza-Derout et al., 2019). Markers of oxidative stress include a decrease in glutathione/glutathionedisulfide (GSH/GSSG) ratio (McGill and Jaeschke, 2015) and an increase in thiobarbituric acid reactive substance (TBARS) (Chen et al., 2018; Kartavenka et al., 2020). Measurement of these markers of oxidative stress in the liver of these chronically e-cig-treated mice remains an important avenue for future investigation.

In conclusion, we used our novel mouse model of e-cig exposure which leads to the plasma nicotine and cotinine levels resembling those in human smokers, and found that 3'-HC and 5'-HC are the major oxidation products of cotinine, and CNO, although less abundant, is measurable in the plasma of these e-cig-treated mice. Accordingly, we propose (3'-HC + 5'-HC + CNO)/Cotinine as an appropriate nicotine metabolite ratio in mice. These three oxidation reactions account for microsomal ROS production which is elevated in the chronically e-cig-treated mice. We have shown that hepatic microsomal CYP2A5 activity and the protein expression in male ppENK heterozygous mice are significantly elevated after repeated exposure to e-cigarettes containing 2.4% nicotine, although deletion of the ppENK gene does not affect CYP2A5 activity. Taken
together, these findings strongly suggest that chronic exposure to nicotine, through ROS-induced oxidative stress, upregulates CYP2A5.

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Authorship contributions

- Participated in research design: Lutfy K, Kanamori K, Ahmad S
- Conducted experiments: Kanamori K, Ahmad S, Lutfy K, Hamid A
- Performed data analysis: Kanamori K
- Wrote or contributed to the writing of the manuscript: Kanamori K, Lutfy K

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Footnote

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Conflict of interest statement:

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Fig. 1. Metabolic pathways of nicotine to cotinine and of cotinine oxidation to trans 3’-hydroxycotinine (3’-HC), 5’-hydroxycotinine (5’-HC) and cotinine N-oxide (CNO) (prepared with ACD Chem-sketch software).

Fig. 2. Coumarin 7’-hydroxylase activity, as the marker of CYP2A5 activity, in the liver microsome of male ppENK (+/-) mice after treatment with e-cig (2.4% nicotine) for 240 min/day for 5 days vs untreated. The data are shown in a box plot with individual activities (n = 4). The median, shown by the line, in the treated (89.35 ng/mL) is higher than that in the untreated (23.85 ng/mL) by Mann-Whitney U test * p = 0.0286.

Fig. 3. Hepatic microsomal coumarin 7’-hydroxylase activity in untreated male mice. The data are the mean ± SD of n = 4 each for ppENK (+/-) and ppENK (-/-) mice and n = 7 for the wildtype. The vertical scale is the same as in Fig. 2 to facilitate comparison. No significant difference was seen in the mean values among these genotypes by one-way ANOVA (p = 0.54).

Fig. 4. Western blots of CYP2A5. A. Representative immunoblots of mouse liver microsomal proteins at the indicated quantities detected selectively at 50 kDa for CYP2A5. B. CYP2A5 density increases linearly with the quantity of microsomal protein. Each data point is the mean ± SD of three independent assays. The error is smaller than the symbol when not shown. C. Upper image. Representative immunoblots of CYP2A5 from each of the four untreated ppENK (+/-) mice (left) and from e-cig-treated ppENK (+/-) mice (right) detected at 50 kDa. Lower image. Immunoblots of GAPDH, as loading control, at 37 kDa. D. CYP2A5 density of untreated vs e-cig-treated shown as dot plots for each mouse. Each data point is the mean of three independent
determinations for each mouse to check reproducibility. The mean density of the e-cig-treated, shown by the horizontal line, is higher than that of the untreated with \( p < 0.05 \) by \( t \)-test (see text for details).

**Fig. 5. A. Upper panel.** The rate of production of DCF, as a measure of ROS, was measured by incubating the liver microsome of a male ppENK (+/-) mouse after chronic treatment with e-cig containing 2.4 % nicotine, with or without (control) 40 \( \mu \)M cotinine. Each data point represents the mean ± sem of \( n = 4 \) assays. The rate of DCF formation due to cotinine oxidation, calculated for the period of \( t = 20-60 \) min, was 21.9 pmol/min/mg microsomal protein. **Lower panel.** Cotinine-induced DCF formation in the microsome of an untreated ppENK (+/-) mouse. Each data point represents the mean ± sem of \( n = 5-6 \) assays. The rate of DCF formation due to cotinine oxidation, calculated for \( t = 20-60 \) min, was 3.2 pmol/min/mg protein. **B.** The rates of cotinine-induced DCF production in male ppENK (+/-) mice after chronic treatment with e-cig (2.4 % nicotine), compared to the untreated. The data are the mean ± SD of \( n = 4 \) for each group. **\( \times \times p = 0.0086 \) by unpaired parametric two-tailed \( t \)-test.

**Fig. 6.** Representative LCMS chromatograms of the plasma samples from a chronic e-cig-treated mouse. **A.** nicotine, detected by MRM of its product ion with m/z of 130.056. **B.** cotinine detected by its parent ion with m/z of 177.1062. **C.** Trans-3’-HC. **D.** 5’-HC and **E.** CNO; all three were detected by their identical parent ion with m/z of 193.0977 but separated by different retention times. Annotation at the peak shows the metabolite name, retention time, and the peak area. The annotation at top right shows the m/z of the detected ion and the peak height. For CNO, the inset shows the peak scaled to
100%. Note that the starting retention time in A and E differs from that in B, C and D, because these were acquired on different days, but the retention time scale is aligned.

**Fig. 7.** A. Nicotine, cotinine, 3’-HC, 5’-HC, CNO, and (3’-HC + 5’-HC + CNO) conc. in the plasma of chronically e-cig-treated mice. The data are the mean ± SD of \( n = 4 \). B. The mean ± SD (\( n = 4 \)) of the ratio of plasma (3’-HC + 5’-HC + CNO) concentration to coumarin 7’-hydroxylase (COH) activity in each mouse (see text for details). C. Correlation between the (3’-HC + 5’-HC + CNO)/cot ratio (NMR) and the coumarin 7’-hydroxylase activity in each of the four chronically e-cig treated mice.
Table 1. LCMS parameters of nicotine and its metabolites

<table>
<thead>
<tr>
<th>Standard compound</th>
<th>RT (min)</th>
<th>[M + H]^+ (m/z)</th>
<th>Product ion (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>3.05</td>
<td>163.1</td>
<td>130.06</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Nicotine-d$_4$</td>
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<td>167.1</td>
<td>134.1</td>
<td>32</td>
<td>20</td>
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<tr>
<td>Cotinine</td>
<td>2.02</td>
<td>177.1062</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotinine-d$_3$</td>
<td>2.02</td>
<td>180.1312</td>
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<tr>
<td>Trans-3’-hydroxycotinine</td>
<td>1.94</td>
<td>193.0977</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Trans-3’-hydroxycotinine-d$_3$</td>
<td>1.96</td>
<td>196.1162</td>
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<td>5’-hydroxycotinine</td>
<td>1.75</td>
<td>193.0977</td>
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<tr>
<td>Cotinine N-oxide</td>
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<td>193.0977</td>
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<tr>
<td>Cotinine N-oxide d$_3$</td>
<td>2.96</td>
<td>196.1173</td>
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</tr>
</tbody>
</table>

The retention time and the m/z of the (a) parent [M+H]^+ ion of cotinine and its oxidation products and (b) of the product ion of nicotine. The parameters for their deuterated isotopomers used as internal standards are also shown (see text for details).
(S)-Nicotine → Other nicotine primary metabolites

CYP2A5

Nicotine- $\Delta^{1'}-5'$-iminium ion

Aldehyde oxidase

(S)-Cotinine

CYP2A5

Trans-3'-hydroxycotinine 3'-HC

5'-hydroxycotinine 5'-HC

(S)-Cotinine-N-oxide CNO

Fig. 1
Fig. 2

Coumarin 7'-hydroxylase

pmol/min/mg

Treatment

None

Chronic e-cig

*
Fig. 3

Coumarin 7'-hydroxylase (pmol/min/mg)

ppK-HET  ppK-KO  WT
Fig. 4

Panel A: Image of a gel with protein bands labeled with different concentrations of microsomal protein (2.4, 3.2, 4.8, 6.4, 9.6, 12.8 μg) and a marker at 50 kDa.

Panel B: Graph showing the relationship between microsomal protein concentration and CYP2A5 density unit. The x-axis represents microsomal protein (μg), and the y-axis represents CYP2A5 density unit.

Panel C: Image of a gel showing bands for untreated ppENK (+/-) and e-cigarette-treated ppENK (+/-) at 50 kDa and 37 kDa, with markers for CYP2A5 and GAPDH.

Panel D: Scatter plot comparing CYP2A5 density unit between untreated and e-cigarette-treated samples. The data points are indicated by circles and a star (*) denotes a significant difference.

Fig. 4
Fig. 6

A

![Graph showing nicotine analysis with peaks at 3.05, 238.72, and 2.99e2 minutes.]

B

![Graph showing cotinine parent with peaks at 2.02, 140553.36, and 1.41e5 minutes.]

C

![Graph showing 3-hydroxy cotinine with peaks at 1.75, 106580.05, and 1.07e5 minutes.]

D

![Graph showing 5-hydroxy cotinine with peaks at 1.75, 182225.55, and 1.82e5 minutes.]

E

![Graph showing CNO with peaks at 2.95 and 72 minutes.]
Fig. 7

A. Plasma concentration (ng/mL) of metabolites.

B. Ratio of (3'-HC + 5'-HC + CNO)/Cot.

C. COH activity (pmol/min/mg) plotted against (3'-HC + 5'-HC + CNO)/Cot.
Chronic exposure to e-cigarettes elevates CYP2A5 activity, protein expression, and cotinine-induced production of reactive oxygen species in mice

Keiko Kanamori, Syed M. Ahmad, Abdul Hamid and Kabirullah Lutfy

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Supplementary Fig. S1. Standard graphs used to calculate metabolite concentration in plasma samples from the observed peak area, or from the response = peak area*IS conc. /IS area where IS is the deuterated internal standard. A. Nicotine, monitored by MRM, showed very similar calibration graphs for the neat standard and for the standard in plasma matrix, so the equation for the former was used to convert the observed response in each plasma sample to nicotine concentration. B. Cotinine standard graph shown as the peak area. C, 3’-HC as the peak area; D, 3’-HC as the response; E, 5’-HC; and F, CNO. Unless otherwise indicated, the standard prepared in the plasma matrix was used to construct the standard graphs.
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Drug Metabolism and Disposition.

DMD-AR-2023-001348

Supplemental data Table S1. GAPDH densities in Fig. 4C (bottom image).

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Density</th>
<th>Mouse ID</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated 1</td>
<td>296020</td>
<td>e-cig-treated 1</td>
<td>134010</td>
</tr>
<tr>
<td>Untreated 2</td>
<td>149129</td>
<td>e-cig-treated 2</td>
<td>123395</td>
</tr>
<tr>
<td>Untreated 3</td>
<td>27100</td>
<td>e-cig-treated 3</td>
<td>81846</td>
</tr>
<tr>
<td>Untreated 4</td>
<td>138276</td>
<td>e-cig-treated 4</td>
<td>65625</td>
</tr>
</tbody>
</table>

The densities of the GAPDH bands were measured on Chemi-Doc XRS+ imager and quantified by ImageLab software (v.6.1). The densities of this loading control in the four e-cig-treated group were lower than those in three of the four untreated mice (The reason for the faint band in untreated mouse 3 is unknown). The result shows that the higher CYP2A5 densities in Fig. 4C (upper image) are not due to loading error, but represents the higher protein levels of this enzyme in the e-cig-treated mice.