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 (e-cigs) (2.4% nicotine). After exposure for 240 minutes per day for 5 days, the activity and the protein level in preproenkephalin (ppENK)-heterozygous [ppENK (+/–)] mice were significantly elevated (P < 0.05) compared with 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 wild-type ppENK (+/+) controls. 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 with 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 1) this metabolite ratio may correlate with plasma nicotine clearance and hence impact nicotine’s psychoactive effects and 2) 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 upregulated by chronic nicotine exposure due to oxidative stress caused by the oxidation of cotinine in this preclinical model of human smokers.

Introduction

Nicotine, the psychoactive ingredient of e-cigarettes (e-cigs), is metabolized to cotinine by CYP2A6 in humans and by its close analog, 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; Muhaisin 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 (Su 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 preproenkephalin (ppENK)-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 minutes per day for 5 days (Results). We have used mice null in the ppENK gene, first characterized by König et al. (1996), to examine the role of enkephalins in morphine tolerance (Marquez et al., 2006), alcohol reward (Tseng et al., 2013), and bingeing (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 wild-type (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 Tynadle, 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 wild-type male mice after e-cig treatment of 1 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 noninvasive 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-Borai and Tynadle, 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), NaNADPH, dimethyl sulfoxide (≥99.5%), (±)-nicotine-d₁ (0.1 mg/ml in acetonitrile), (±)-cotinine-d₁ (1 mg/ml in methanol), trans 3'-hydroxycotinine (1 mg/ml in methanol), trans 3'-hydroxycotinine-d₁ (0.1 mg/ml in methanol), coumarin, 7'-hydroxycoumarin, and Bradford reagents were purchased from MilliPersigma (St. Louis, MO). (-)-Cotinine was purchased from Cayman Chemical (Ann Arbor, MI). 5'-Hydroxycoumarin (N-methyl-2-oxo-3-pyridinebutanamide) was purchased from Santa Cruz Biotechnology (Dallas, TX). (S)-cotinine N-oxide and (R)-cotinine-d₁ 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 mass standard (1610373) were purchased from Bio-Rad Laboratories (Hercules, CA).

Primary antibody to human CYP2A6 (ab3570), primary antibody to glyceraldehyde 3-phosphate dehydrogenase (ab9485), horseradish peroxidase-conjugated secondary antibody (ab205718) and enhanced chemiluminescence substrate kit (ab34406) 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 wild-type (n = 7) and heterozygous littermates/age-matched controls (n = 8) were bred in house. Mice were fully backcrossed on a C57BL/6d mouse strain and were maintained on a 12-hour light/12-hour dark cycle (light on at 06:00). All experiments were carried out according to National Institute of Health (NIH) guidelines for the proper care and use of animals in research and approved by the Institutional Animal Care and Use Committee (211AACUC/009) at the Western University of Health Sciences (Pomona, CA).

Four ppENK (+/-) mice were used for chronic e-cig treatment. Each mouse was brought to the laboratory and habituated to the room for 1 hour. Mice were then exposed to an e-cig containing 2.4% nicotine for 4 hours (from 10:00 to 14:00). The e-cig system, EicigAero, manufactured by AutoMate Scientific, Inc. (Berkeley, CA) and 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., 10 puffs per hour, with a puff duration of 4 seconds and interpuff interval of 10 seconds) using Classic Tobacco flavor blu 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 that 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 to 6 minutes after the 4-hour exposure period. The trunk blood was collected in 1.5-ml vials containing 50 μl of a 7% ethylenediamine tetracetic acid (EDTA) solution. The vials were then spun for 10 minutes 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/6d wild-type 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 minutes to precipitate cell debris, and this procedure was repeated twice. The supernatant was then centrifuged at 38,700 x g for 20 minutes. 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 minutes. 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 a BioTek Synergy II Multimode Reader (Winooski, VT). The fluorescence in the assay mixture without incubation (control) was subtracted from the fluorescence in the incubated mixture and 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 NaHPO₄ buffer (pH 7.4) to achieve a concentration of 6.4 nl protein/10 μl, followed by the addition of 10 μl of the sample loading buffer and heating at 70°C for 10 minutes 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 hour 50 minutes. For the detection of CYP2A5, nitrocellulose membranes were preincubated for 1 hour 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 (1:3000 dilution) and a horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping protein, was used as the loading control and probed with the primary antibody at 1:10,000 dilution followed by incubation with the secondary antibody. The molecular masses of the detected CYP2A5 and GAPDH bands were confirmed by running molecular mass 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 mass standards by colorimetric assay followed by incubation with a Bio-Rad ChemiDoc XRS+ image system. The protein density was quantified by Bio-Rad Image Laboratory 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 al., 1983) 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) and 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 that were essential for successful assay in our laboratory. Cotinine [400 μM in Na2HPO4 buffer (pH 7.4)], NADPH [10 mM in Na2HPO4 buffer (pH 7.4)], and DCFH-DA (250 μM) were freshly prepared 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 minutes at 37°C to allow uptake of the nonfluorescent 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. A 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 a BioTek Synergy II Microplate Reader 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 minutes for 1 hour 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. The mean value of pmol/mg protein was then 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 and

![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).](image-url)
then calculating the rate over the period of linear increase. The two methods provided virtually the same rate of cotinine-induced DCF production.

**Liquid Chromatography Mass Spectrometry Assay of Plasma Nicotine and Its Metabolites.** Liquid chromatography mass spectrometry (LCMS) experiments were performed on an ACQUITY UPLC system coupled to a Xevo Time-of-Flight mass spectrometer (Waters Corporation, Milford, MA), 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 mass-to-charge ratio (m/z) of 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 Fig. 1. 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 Cotinine 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 masses of cotinine versus 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**

**Cotumin 7'-Hydroxylase Activity in Chronic E-Cig–Treated Mice.** Figure 2 shows cotumin 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 minutes per 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 ng/ml, with P = 0.0286 by Mann-Whitney U test. The result shows that chronic e-cig treatment elevates cotumin 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 wild-type and heterozygous littermates/controls. Figure 3 compares cotumin 7'-hydroxylase activities, as the mean ± S.D., in the liver microsomes of untreated male ppENK (+/−) (n = 4), ppENK (−/−) (n = 4), and wild-type 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.** Figure 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**). Figure 4B plots CYP2A5 density as the mean ± S.D. of three independent assays; the density increases linearly with protein over 2.4 to 12.8 μg. Figure 4C (upper image) shows a representative immunoblot of liver microsomal CYP2A5 from each of the four untreated ppENK(+/−) mice (left four lanes) and from the e-cig–treated ppENK(+/−) mice (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 was 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 housekeeping protein with a molecular mass 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 Table 1; 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 with the untreated. Figure 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, df = 6). The result shows that repeated nicotine treatment via e-cig exposure elevates the protein level of liver microsomal CYP2A5.

**Cotumin-Induced ROS Production.** Figure 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 μM cotinine. The rate of DCF formation was linear during t = 20–60 minutes and was faster in the presence of cotinine than in its absence (control) (n = 4 assays per

### TABLE 1

LCMS parameters of nicotine and its metabolites

<table>
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<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>
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<tr>
<td>Nicotine</td>
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<td>163.1</td>
<td>130.06</td>
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<td>20</td>
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<tr>
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<td>134.1</td>
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<td>177.1062</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotinine-d4</td>
<td>2.02</td>
<td>180.1312</td>
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<tr>
<td>Trans-3'-hydroxocotinine</td>
<td>1.94–1.95</td>
<td>193.0977</td>
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</tr>
<tr>
<td>Trans-5'-hydroxocotinine-d3</td>
<td>1.96</td>
<td>196.1162</td>
<td>193.0977</td>
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<tr>
<td>5'-Hydroxocotinine</td>
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<td>193.0977</td>
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<td>193.0977</td>
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*eV, electron volt; RT, retention time.*

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data point, \( P < 0.01 \) at \( t = 20–30 \) minutes, \( P < 0.05 \) at \( t = 35–60 \) minutes 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). Figure 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 ± S.D. of four animals per 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 (\( t = 3.84, df = 6 \)).

Fig. 2. Coumarin 7′-hydroxylase activity, as the marker of CYP2A5 activity, in the liver microsomes of male ppENK (+/−) mice after treatment with e-cig (2.4% nicotine) for 240 minutes per day for 5 days versus 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. 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 μM cotinine. Each data point represents the mean ± S.E.M. of n = 4 assays. The rate of DCF formation due to cotinine oxidation, calculated for the period of t = 20–60 minutes, 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 ± S.E.M. of n = 5 to 6 assays. The rate of DCF formation due to cotinine oxidation, calculated for t = 20–60 minutes, 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 with the untreated. The data are the mean ± S.D. of n = 4 for each group. **P = 0.0086 by unpaired parametric two-tailed t test.

Another notable feature is that the substrate cotinine shows little interanimal variability; the coefficient of variation, viz (the standard deviation/mean) × 100% is 13.3%. By contrast, there is high interanimal variability; the coefficient of variation of 42%. To examine whether this interanimal 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 ± S.D. 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 interanimal 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'-HC + 5'-HC + CNO)/cotinine. Figure 7C plots (3'-HC + 5'-HC + CNO)/cotinine in molar ratio (Materials and Methods) in each mouse versus 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'-HC + 5'-HC + 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 wild-type 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'-HC + 5'-HC + CNO)/Cot, is highly correlated with hepatic microsomal CYP2A5 activity. The mean ± S.D. of the nicotine metabolite ratio/COH activity was 0.01826 ± 0.00484 minutes· 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 et al. (2017) 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. 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 ID13087 located on chromosome 7) as was not different among untreated ppENK (+/−) and their ppENK (−/−) and wild-type littermates (Fig. 3). The protein level of CYP2A5, measured by western blot, was higher in the four e-cig-treated ppENK (+/−) mice compared with 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 minutes per day for 5 days). By contrast, in male wild-type mice exposed to e-cigarettes with the same nicotine dose for only 1 day (n = 3), the microsomal CYP2A5 activity did not differ from that in untreated wild-type 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 were incubated with cotinine compared with that in untreated ppENK (+/−) mice (Fig. 5, A 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 4-fold higher in microsomes from CYP2A5/+− wild-type mice compared with those from CYP2A5−/− 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 microsomal ROS production (in pmol/min/mg) in chronically e-cig-treated male mice versus the untreated counterparts (Fig. 5B). Third, 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-D1A-D2-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-cigarette–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 and 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 minutes (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 noninvasive 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 physiologic perturbation, for example, by tail vein blood sampling, or noninvasively 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 noninvasive 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 (Abubakar et al., 2013; Muhaisen 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 antioxidant 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; Kartavenko 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 chronic 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 that 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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Data Availability

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

Authorship Contributions

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

References

Kanamori K, Ahmad SM, Shin CS, Hamid, and Lutfy K (2022) Identification of 5-hydroxycytosine nine in smoke 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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