Chemical Research in Toxicology

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Electronic Cigarette Solvents, JUUL E-Liquids, and Biomarkers of Exposure: In Vivo Evidence for Acrolein and Glycidol in E-Cig-Derived Aerosols

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Cite This: *Chem. Res. Toxicol.* 2022, 35, 283−292

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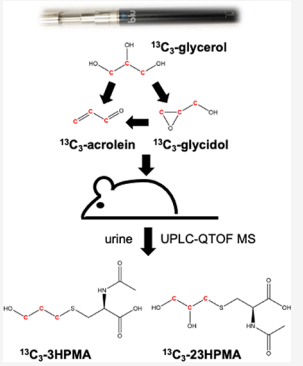
ABSTRACT: Despite the increasing popularity of e-cigarettes, their long-term health effects remain unknown. In animal models, exposure to e-cigarette has been reported to result in pulmonary and cardiovascular injury, and in humans, the acute use of e-cigarettes increases heart rate and blood pressure and induces endothelial dysfunction. In both animal models and humans, cardiovascular dysfunction associated with e-cigarettes has been linked to reactive aldehydes such as formaldehyde and acrolein generated in e-cigarette aerosols. These aldehydes are known products of heating and degradation of vegetable glycerin (VG) present in e-liquids. Here, we report that in mice, acute exposure to a mixture of propylene glycol:vegetable glycerin (PG:VG) or to e-cigarette-derived aerosols significantly increased the urinary excretion of acrolein and glycidol metabolites—3-hydroxypropylmercapturic acid (3HPMA) and 2,3-dihydroxypropylmercapturic acid (23HPMA)—as measured by UPLC-MS/MS. In humans, the use of e-cigarettes led to an increase in the urinary levels of 23HPMA but not 3HPMA. Acute exposure of mice to aerosols derived from PG:$^{13}C_3$-VG significantly increased the $^{13}C_3$ enrichment of both urinary metabolites $^{13}C_3$-3HPMA and $^{13}C_3$-23HPMA. Our stable isotope tracing experiments provide further evidence that thermal decomposition of vegetable glycerin in the e-cigarette solvent leads to generation of acrolein and glycidol. This suggests that the adverse health effects of e-cigarettes may be attributable in part to these reactive compounds formed through the process of aerosolizing nicotine. Our findings also support the notion that 23HPMA, but not 3HPMA, may be a relatively specific biomarker of e-cigarette use.



1. INTRODUCTION

Tobacco smoke is the single-most significant modifiable risk factor in the development of cardiovascular disease (CVD).[1,2] Exposures to both mainstream[3,4] and secondhand[3,5] cigarette smoke increase the risk for CVD. Although smoking combustible cigarettes clearly increases the risk of heart attack, coronary artery disease, atherosclerosis, and stroke,[6] there are no studies that link the use of e-cigarettes with major adverse cardiovascular events. However, the results of many studies show that in healthy adults, the use of e-cigarettes leads to an acute increase in heart rate and blood pressure as well as decrements in flow-mediated dilation,[7] which is indicative of significant endothelial dysfunction.[6] Endothelial dysfunction is *sine qua non* in atherosclerosis, and it serves as an early, sensitive, and specific biomarker of cardiovascular harm, predictive of future cardiovascular events.[8]

Nonetheless, the constituents of e-cigarettes as well as their mechanisms that mediate cardiovascular dysfunction have not been identified. Previous work has shown that e-cigarette aerosols, like mainstream or side-stream cigarette smoke, contain measurable amounts of reactive carbonyls such as acrolein, formaldehyde, and acetaldehyde.[9,10] A report from the Institute of Medicine ranked acrolein, formaldehyde, and acetaldehyde as three of the most significant toxins in mainstream tobacco smoke particularly in relation to non-cancer disease risk, i.e., CVD.[11] Estimates of the relative toxicity of different constituents of combustible cigarette aerosols indicate that more than 80% of the noncancer risk of smoking could be attributed to carbonyls such as acrolein.[11] Significant levels of acrolein and other unsaturated and saturated aldehydes have also been detected in e-cigarette aerosols, and the extent of generation of these aldehydes depends upon the operating conditions of the e-cigarette device (e.g., wattage), user topography, and the relative abundance of propylene glycol (PG) and vegetable glycerin (VG) in the e-liquid.[12−18] Regardless of differences in e-cigarette constituents or use conditions, toxic aldehydes such

Received: September 28, 2021
Published: January 19, 2022

as acrolein are generated during the aerosolization of nicotine solutions.

In recent years, several groups show that aldehydes generated in e-cigarette aerosols are derived mainly from thermal degradation of VG (or PG).[18−20] This is not surprising—the phenomenon of thermal VG breakdown into acrolein (and formaldehyde) was first described in the 19th century,[21] and more recent studies further delineate pathways of degradation in the gas phase under various conditions.[22,23] Notably, as outlined by Laino *et al.*, the first and limiting step in the process of VG dehydration is formation of the epoxide, glycidol, that can undergo further conversion to formaldehyde or to acrolein—the latter, a more energetically demanding process, facilitated by heating and acidic conditions.[22] Interestingly, the latter reaction is widely used in high-scale acrolein manufacturing, where the glycerin substrate undergoes dehydration under high temperatures and in the presence of zeolite catalysts.[24] Taken together, VG is a well-documented source of acrolein, formaldehyde, and other reactive compounds like glycidol in e-cigarette aerosols.[19,25] The formation of various aldehydes during e-cigarette use is also confirmed by measurements in e-cigarette aerosols exhaled by human subjects.[26]

Studies of human e-cig users report widely varying levels of urinary 3-hydroxypropylmercapturic acid (3HPMA) that are typically much lower than urinary levels of 3HPMA in combustible cigarette users,[27−30] whereas an e-cigarette aerosol exposure in mice increases the urinary level of 3HPMA.[17] Nevertheless, it is unclear whether higher levels of 3HPMA in the urine after e-cigarette aerosol exposure are derived from e-cigarette aerosols. In both mice and humans, acrolein is generated endogenously from a variety of biological reactions including lipid peroxidation and myeloperoxidase-catalyzed reactions.[31,32] Acrolein is also present in many different foods,[33] and thus, an increase in the levels of 3HPMA in e-cigarette users may be secondary to other sources including diet, intermediary metabolism, inflammation, or oxidative stress. Thus, to identify the source of acrolein that contributes to urinary 3HPMA in e-cigarette users, we exposed mice to $^{13}C_3$-VG and probed for ^{13}C-enrichment in urinary metabolites of acrolein and glycidol using UPLC-QTOF mass spectrometry.

2. EXPERIMENTAL PROCEDURES

2.1. Materials. Unless otherwise stated, reagent-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO). UHPLC/UHPLC−MS-grade solvents—water (Honeywell), acetonitrile (Thermo Fisher), and methanol (Thermo Fisher)—were purchased from Fisher Scientific (Chicago, IL). Analytical standards—2,3-dihydroxypropylmercapturic acid (23HPMA), D_5-23HPMA, and 3-hydroxypropylmercapturic acid (3HPMA), D_3-3HPMA—were obtained from Toronto Research Chemicals (Toronto, CAN). For purposes of harmonizing acronyms/abbreviations of metabolites of common volatile organic compounds (VOCs), we have adopted the naming convention put forward by Tevis *et al.*[34]

2.2. Mice and Exposures. *2.2.1. Mice.* Male C57BL/6J (wild type, WT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were treated according to the *Guiding Principles for the Care and Use of Animals in Research and Teaching* as adopted by the American Physiological Society, and all protocols were approved by the University of Louisville Institutional Animal Care and Use Committee. Before and during exposures, mice were housed under pathogen-free conditions, controlled temperatures, and a 12 h:12 h light:dark cycle. Mice were maintained on a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

2.2.2. E-Cigarette Aerosol Exposures. A software-controlled (FlexiWare) cigarette-smoking robot (SCIREQ; Montreal, CAN) system was used in the mechanical generation of aerosols from JUUL e-liquids or PG:VG mixtures. To control the generation of volatile organic compounds (VOCs) in e-cig aerosols, we used a defined e-cig platform as described.[35] A JUUL e-liquid (Virginia Tobacco, Menthol, Mango; pods purchased online) or a PG:VG mixture (50:50 or 30:70 ratio, v:v) was loaded into a refillable, clear tank atomizer with a fixed coil resistance (Mistic Bridge; approximately 2.4 ohm; purchased online) coupled with a rechargeable bluPLUS+ (3.7 V; purchased online) battery (power output of approximately 6 W; Figure S1), which is comparable relatively with the power of a JUUL device.[17] The atomizer tank was weighed before and after use to quantify solution consumption (mg/puff). A 9 min session entailed murine exposure to 18 puffs (4 s/puff, 91.1 mL/puff, 2 puffs/min). In a 6 h exposure, 20 sessions were evenly spaced. Total suspended particulate (TSP) matter was monitored in real time with an inline infrared Microdust Pro 880 nm (Casella) probe positioned upstream of the octagon exposure chamber (5 L; SCIREQ). Mice were exposed to e-cigarette aerosols between 7 A.M. and 2 P.M. in the absence of food or water.

2.3. Urine Collection and Metabolism. *2.3.1. Urine Collection: Murine Study.* Prior to exposures, mice were held and a small drop of D-glucose:saccharin solution (3.0%/0.125% w/w; Sigma-Aldrich; St. Louis, MO) was touched to their mouth. For the ^{13}C-VG study, we mixed PG (1.0 mL), VG (0.8 mL), and ^{13}C-VG (0.2 mL) for a final 50:50 (PG:VG) ratio and exposed mice to aerosols for 6 h. After 6 h exposures (air or PG:^{13}C-VG), mice were placed singly per metabolic cage (Harvard Apparatus; Cambridge, MA) for urine collection without food yet with access to glucose:saccharin drinking water. Urine was collected in graduated cylinders surrounded by 4 °C water-jacketed organ baths from 0 to 3 h post exposure, as well as in a second overnight collection (3−16+ h, O/N) during which mice were provided both glucose/saccharin solution and food.[36] Urine samples were centrifuged (1800*g*, 5 min; to pellet feces or food) before being decanted and stored at −80 °C.

2.3.2. Urine Collection: Human Study—E-Cigarette Vascular Assessment (EVA) (University of Louisville, IRB:16.0685). Nine infrequent e-cig product users (1 or fewer times per day) were recruited for the study. The participants were asked to abstain from smoking, vaping, and tobacco use of any kind 12 h prior to the visit. Eight hours before the visit, participants were asked to fast and avoid any caffeinated beverages, alcohol, and fried food. Clean catch urine was collected immediately prior to the product use (i.e., "0 h"). For exposure, the participants were asked to use their own e-cig product as they normally would and produce at least 15 puffs. All nine participants used a mod-type device (third gen device) with a refillable tank and their own e-liquid. The maximum exposure time was no longer than 15 min. Four subsequent urine samples within the 3 h period were collected with the first collection immediately after the exposure. To aid the production of urine, participants were instructed to drink water *ad libitum*. The 23HPMA metabolite was measured in user specimens at all time points.

2.3.3. Urine Collection: Human Study—Reactive Aldehydes in Tobacco Study (RATS) (University of Louisville, IRB:15.0097). Urine sample collection and the study protocol were described previously.[29] Briefly, a clean catch urine sample was obtained from the participants who were instructed to abstain for 48 h from tobacco, e-cigarettes, nicotine, and smoking of any kind (including marijuana and other illicit drugs). These frequent tobacco product users also were asked not to eat and drink any caffeinated or alcoholic beverages or grapefruit juice 8 h prior to the second visit. Immediately after urine collection, the participants used the tobacco product. Depending upon the study group, participants were asked to smoke one Marlboro Red cigarette (nicotine, 1.2 mg/cigarette) or NJOY King e-cigarette (2.4% nicotine). E-cig products were used *ad libitum* but not longer than 15 min and not less than 15 puffs. A fresh urine sample was obtained 20 min (±5 min) after the first collection. Thus, urine was collected at specific time points: immediately before exposure (0) and at 20, 40, 80, 120, and 180 ± 5 min after the first urine sample. The

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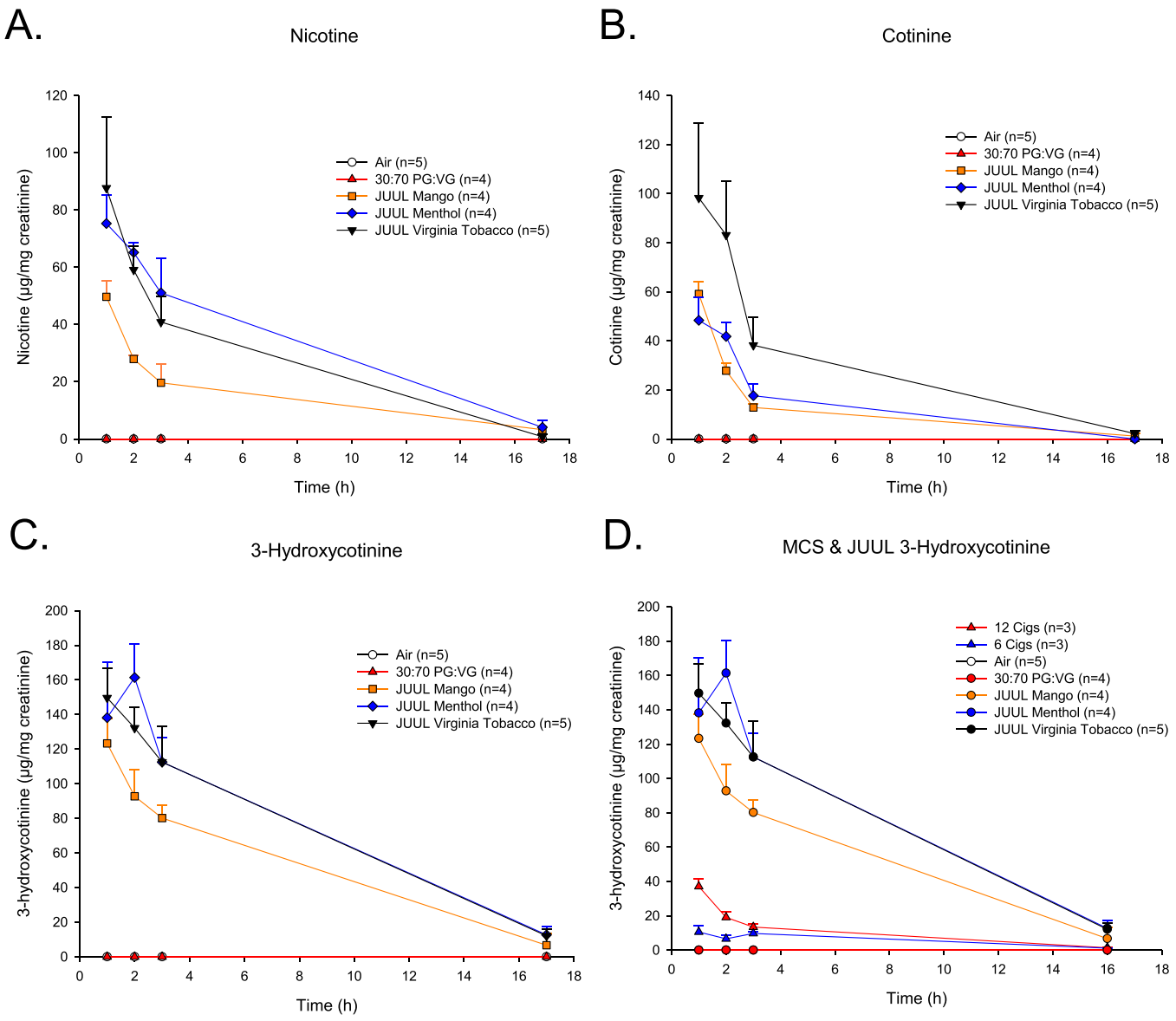

Figure 1. Nicotine metabolism and excretion kinetics in PG:VG- and JUUL-exposed mice. Urinary levels of (A) nicotine, (B) cotinine, and (C) *trans*-3-hydroxycotinine at 1, 2, 3, and 3−17 h after a 6 h exposure of male C57BL6J mice to filtered air, propylene glycol:vegetable glycerin (PG:VG; 30:70)-derived aerosols, or JUUL e-liquid-derived aerosols. (D) Urinary levels of *trans*-3-hydroxycotinine in mainstream cigarette smoke (MCS; 3R4F; 50% of the smoke of 6 or 12 cigarettes) at 1, 2, 3, and 3−17 h after a 6 h exposure (for comparison with exposures to PG:VG- or JUUL e-liquid-derived aerosols). Values = mean ± SEM (n = 3−5 male mice per group).

23HPMA metabolite was measured in a randomly chosen subset of e-cig and combustible cigarette (N = 5) user specimens at all time points.

2.3.4. Urine Metabolite Analysis. 2.3.4.1. ^{13}C-Labeled Metabolite Discovery/Identification. LC-HRMS analysis was performed on a Waters Synapt XS HDMS coupled with an ACQUITY UPLC I-Class system. Separation was carried out on the Acquity Premier CSH C18 column (150 mm × 2.1 mm, 1.7 µm). Mouse urine (25 µL) was diluted (10×) with solvent A. The separation was performed using a binary gradient with 0.1% formic acid in UHPLC-grade water (Honeywell) as solvent A and 0.1% formic acid in acetonitrile as solvent B (UHPLC−MS, Thermo Scientific). Gradient conditions: 0.0−11.0 min, 100−77% A; 11.0−14.6 min, 77−5% A; 14.6−17.0 min, 5% A; and 17.05−20.0 min, 100% A. The following settings were used: flow rate, 0.5 mL/min; sample injection volume, 1 µL; column temperature, 50 °C; sample temperature, 5 °C. Synapt XS HDMS data were acquired in the negative ion MSe mode. Authentic standards of 3HPMA and 23HPMA in water (100 ng/mL) were also prepared and analyzed using identical conditions to confirm and validate the assignments in urine samples (retention time, MS/MS, and external database match). The Waters UNIFI software package was used for data analysis and metabolite identification. The El-Maven and PollyPhi packages (Elucidata, MA) were used to assign ^{13}C-labeled isotopologues, as well as to correct for the natural abundance of ^{13}C, as described previously.[37]

2.3.4.2. Quantification of 23HPMA in Human Urine. An Agilent 6460 triple quadrupole mass spectrometer with an Agilent Jet Stream ESI ion source coupled with an Agilent 1290 Infinity II UHPLC system was used for quantitative LC−MS/MS analysis of the glycidol metabolite 23HPMA. Ion source parameters were as follows: nebulizing gas pressure, 50 psi; sheath gas flow, 11 L/min; temperature, 350 °C; drying gas flow, 11 L/min; temperature, 290 °C; capillary voltage (capillary entrance), 3000 V; nozzle voltage, 1500 V (in negative mode). Three multiple reaction monitoring (MRM) transitions were set up for metabolite quantification and measures of the internal standard (IS): ESI quantification transition, 236/107 (collision energy (CE) at 12 V); confirmation transition, 236/128 (CE at 4 V); IS transition, 241/112 (CE at 12 V). All three

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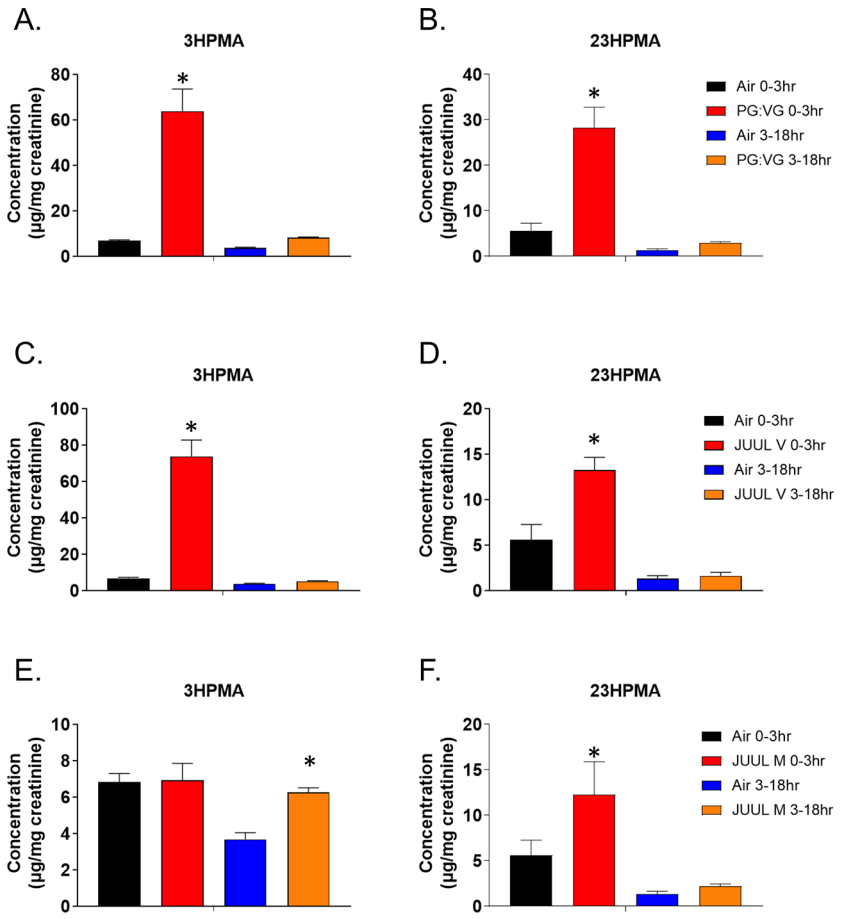

Figure 2. Excretion kinetics of acrolein and glycidol metabolites in PG:VG- and JUUL e-liquid-derived aerosol exposed mice. (A, B) Urinary levels of 3-hydroxypropylmercapturic acid (3HPMA) and 2,3-dihydroxypropylmercapturic acid (23HPMA), respectively, at 0−3 and 3−18 h after a 6 h exposure of female C57BL6J mice to filtered air or PG:VG-derived (30:70) aerosols. (C, D) Urinary levels of 3HPMA and 23HPMA, respectively, at 0−3 and 3−18 h after a 6 h exposure of female C57BL6J mice to filtered air or JUUL Virginia Tobacco (JUUL-V) e-liquid-derived aerosols. (E, F) Urinary levels of 3HPMA and 23HPMA, respectively, at 0−3 and 3−18 h after a 6 h exposure of female C57BL6J mice to filtered air or JUUL Menthol (JUUL-M) e-liquid-derived aerosols. Values = mean ± SEM (n = 3−5 female mice per group); *p < 0.05 vs matched air control.

transitions were with fragmentary voltage (capillary exit) at 83 V. Human urine (100 μL) was diluted (5×) with solvent A and spiked with the D_5-23HPMA standard. Separation was performed on the Waters Acquity HSS T3 column (150 mm × 2.1 mm, 1.8 μm) (Waters Inc.) at 40 °C using 5 μL injection and binary gradient elution composed of solvent A−0.05% formic acid in UHPLC-grade water (Honeywell) and solvent B−methanol (UHPLC−MS, Thermo Scientific), delivered at a flow rate of 0.36 mL/min. Gradient conditions were as follows: 0.0−0.6 min, 2−5% B; 0.6−2.5 min, 5−18% B; 2.5−9.0 min, 18−98% B; 9.0−10.0 min, 98% B; 10.1−12.0 min, 2% B. MassHunter software (Agilent) was used for peak integration, calibration, and quantification. 23HPMA was quantified using the peak area ratio based on nine-point standard curves, which were run before and after the urine samples. The concentrations of 23HPMA were normalized to creatinine levels measured on a COBAS MIRA-plus analyzer (Roche, NJ) with Infinity Creatinine Reagent (Thermo Fisher Scientific).

2.3.4.3. Quantification of 3HPMA and Tobacco Alkaloids in Human Urine. For UPLC-MS/MS analysis, urine samples were diluted with solvent A of UPLC gradient with isotopically labeled internal standards and then applied on an UPLC-MS/MS instrument (ACQUITY UPLC core system and a Quatro Premier XE triple quadrupole mass spectrometer with an electrospray source, all from Waters Inc.). Samples were separated on an Acquity UPLC HSS T3 (150 mm × 2.1 mm, 1.8 μm) column (Waters Inc.) with a binary gradient (solvent A was 15 mM ammonia acetate (pH 6.8) and solvent B was acetonitrile) at a flow rate of 0.45 mL/min. Optimized cone voltage and collision energy were used for each individual analyte. For each analyte, three multiple reaction monitoring (MRM) transitions were set up: one for quantification, one for confirmation, and one for the labeled internal standard. These MRMs were scheduled around the retention time of the analytes. Analytes in urine samples were quantified using the peak area ratio based on 10-point standard curves, which were run before and after the urine samples. The TargetLynx quantification application manager software (Waters Inc.) was used for peak integration, calibration, and quantification. The concentration values of analytes were normalized to the creatinine level, which was measured on a COBAS MIRA-plus analyzer (Roche, NJ) with Infinity Creatinine Reagent (Thermo Fisher Scientific).

2.3.4.4. Quantification of Formate and Acetate. Urinary levels of formate and acetate, the primary metabolites of FA and AA, respectively, were measured by gas chromatography−mass spectrometry (GC−MS) as adapted and modified from previous reports.[38,39] Urine (20 μL) was mixed with sodium phosphate (20 μL; 0.5 M, pH 8.0) containing ^{13}C-formate (2.3 mM) and ^{13}C-acetate (0.23 mM) internal standards and pentafluorobenzyl bromide (130 μL, 0.1 M). The mixture was vortexed for 1 min and then incubated at 60 °C for 15 min, and the resulting reaction products were extracted using hexane (300 μL) before being transferred to glass tubes for GC−MS analysis. Analytes in urine samples were quantified using the peak area ratio based on 7-point standard curves that were run before and after the urine samples. MassHunter software (Agilent) was used for peak integration, calibration, and quantification. Measured formate and

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Table 1. Urine 3HPMA Levels Normalized to Total Nicotine Equivalents (TNE) across JUUL E-Liquids and Mainstream Cigarette Smoke (MCS) Exposures in Male Mice[a]

product	3HPMA [ng/mL]	Nic [nmol/mL]	Cot [nmol/mL]	3HC [nmol/mL]	TNE (Nic + Cot + 3HC)	3HPMA/TNE [ng/nmol]
HEPA	2224.29 ± 1161.45	–	–	–	–	–
JUUL Mango	11026.25 ± 98.94$^{\&\$}$	25.87 ± 4.09	23 ± 4.47	55.19 ± 4.80$^{\$}$	101.9 ± 7.47$^{\$}$	97.68 ± 2.79$^{\&\$\%}$
JUUL Menthol	6761.23 ± 62.16$^{\&\$}$	48.12 ± 6.57	26.46 ± 4.99	81.27 ± 8.15	155.85 ± 11.42	48.46 ± 1.68$^{\&\$}$
JUUL-V	13401.43 ± 120.81$^{*\&\$}$	33.00 ± 4.63	32.50 ± 4.62	63.20 ± 6.58$^{\$}$	128.71 ± 8.93	86.21 ± 4.64$^{\&\$}$
MCS (6 cigs$^{\#}$)	115752.57 ± 167.54*	56.94 ± 10.03	16.35 ± 2.76	86.76 ± 7.47	160.04 ± 7.31	843.64 ± 13.91
MCS (12 cigs$^{\#}$)	152416.50 ± 383.57*	373.00 ± 41.93	48.08 ± 7.76	277.47 ± 17.28	698.54 ± 37.50	347.22 ± 8.85$^{\&}$
p	<0.001	0.527	0.585	0.012	0.029	<0.001

[a]Values = mean ± SEM (n = 3−5 male mice per group). Abbr.: Nic, nicotine; Cot, cotinine; 3HC, *trans*-3′-hydroxycotinine; HEPA, filtered air control; −, not detected. The superscript number sign (#) represents 50% of the smoke generated per number of 3R4F cigarettes. Values (3HPMA only) were log-transformed for normality. P-values based on ANOVA with Tukey adjustment for multiple comparisons: asterisk symbol (*), significant difference from HEPA; superscript ampersand symbol (&), significant difference from MCS (6 cigs); superscript dollar sign ($), significant difference from MCS (12 cigs); superscript (%), significant difference from JUUL Menthol e-liquid.

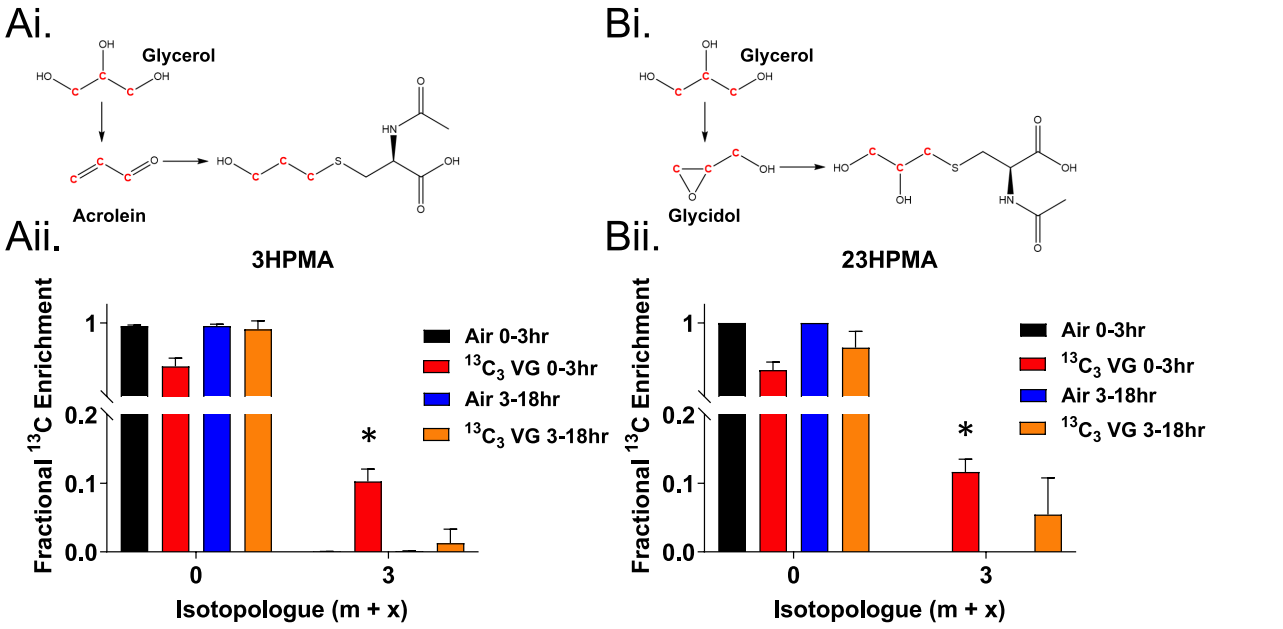

Figure 3. Fractional enrichment of $^{13}C_3$ in urinary metabolites following PG:$^{13}C_3$-VG exposure in mice. (Ai) Chemical structures of parent $^{13}C_3$-glycerol (^{13}C atoms in red), acrolein, and 3-hydroxypropylmercapturic acid (3HPMA). (Aii) Fractional enrichment of urinary 3HPMA isotopologues at 0−3 and at 3−18 h after a 6 h exposure of male C57BL6J mice to filtered air or PG:$^{13}C_3$-VG-derived (50:50) aerosol. (Bi) Chemical structures of parent $^{13}C_3$-glycerol (^{13}C atoms in red), glycidol, and 2,3-dihydroxypropylmercapturic acid (23HPMA). (Bii) Fractional enrichment of urinary 23HPMA isotopologues at 0−3 and 3−18 h after a 6 h exposure of male C57BL6J mice to filtered air or PG:$^{13}C_3$-VG-derived aerosol. Note that $^{13}C_3$-VG represented 10% of the total PG:VG (by volume) and 20% of the VG (by volume). Values = mean ± SEM (n = 8 male mice per group). *, significant difference from matched air control.

acetate sample concentrations were corrected for the natural abundance of the ^{13}C isotopes and normalized to urinary creatinine.[17] Additionally, in studies with mice, we estimated the total excreted formate and acetate by multiplying the measured concentrations (ng/mL) and the total urine volume (mL) collected at each time point and then summed over all time points of the post-exposure interval (0−3 h, O/N).

2.4. Statistics. Data are presented as mean ± standard error of mean (SEM). For comparing two groups, we used the rank sum test with Bonferroni's post-test or paired (or one-way repeated measures ANOVA) or unpaired t-tests as appropriate. For multiple group comparisons, we used one-way ANOVA with Bonferroni's or Tukey adjustments or when variation indicated ANOVA on logarithm-normalized data for multiple comparisons (SigmaPlot, ver. 12.5; Systat Software, Inc., San Jose, CA). Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Murine Study. Mice exposed (6 h) to aerosols derived from JUUL e-liquids excreted high concentrations of urine nicotine, cotinine, and *trans*-3-hydroxycotinine within 1 h post exposure, which decreased progressively over the next 18 h (Figure 1A−C). The JUUL e-liquids used (Virginia Tobacco, Mango, Menthol) produced similar profiles of urinary metabolite excretion, indicating that our exposure platform and conditions produce similar exposures. For context, mice exposed to the smoke of 3R4F Kentucky Reference cigarettes (50% of the smoke of either 6 or 12 cigarettes over 6 h) excreted a fraction of *trans*-3-hydroxycotinine (25−30%) compared with that excreted by mice exposed to JUUL e-liquid-derived aerosols, indicating that exposure to e-cigarette aerosols leads to significantly higher excretion levels of nicotine (and its metabolites) than combustible cigarettes under these conditions as normalized to creatinine (Figure 1D). As

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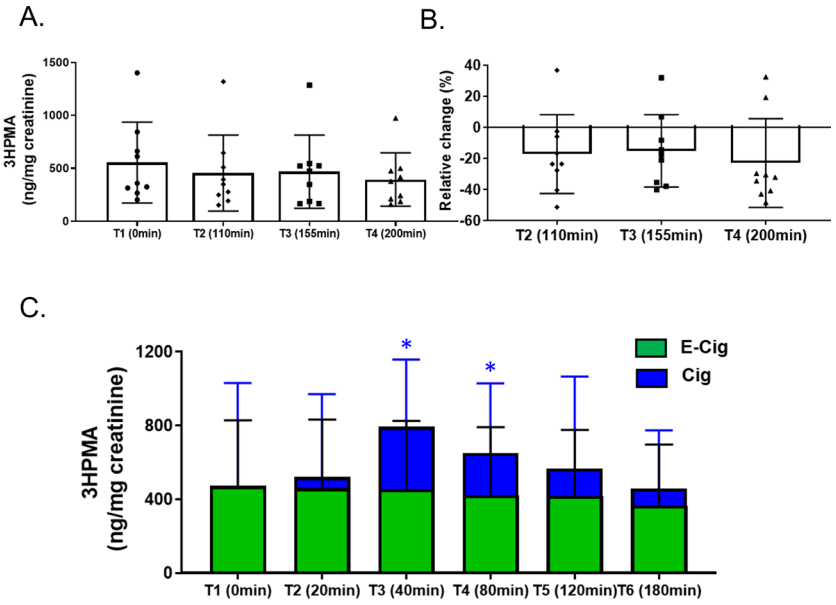

Figure 4. Excretion kinetics of acrolein metabolite (3HPMA) in e-cig users. (A) Urinary levels of 3-hydroxypropylmercapturic acid (3HPMA, ng/mg creatinine) at 0, 110, 155, and 200 min after an acute use of e-cigs (n = 9 EVA study participants). (B) Relative change (from baseline) of urinary 3HPMA at 110, 155, and 200 min after an acute exposure to e-cig-derived aerosols (n = 9 EVA study participants). (C) Urinary levels (ng/mg creatinine) of 3HPMA at 0, 20, 40, 60, 80, 120, and 180 min after an acute use of e-cig or combustible cigarettes (cig) (n = 5 RATS study subjects per group). Values = mean ± SEM. *p < 0.05 vs T1 (0 min) baseline.

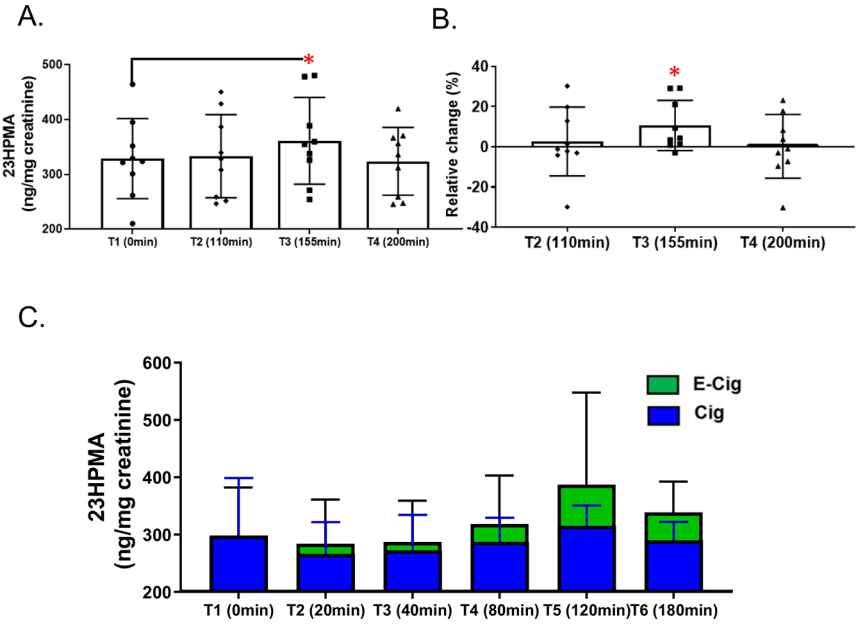

Figure 5. Excretion kinetics of glycidol metabolite (23HPMA) in e-cig users. (A) Urinary levels of 2,3-hydroxypropylmercapturic acid (23HPMA, ng/mg creatinine) at 0, 110, 155, and 200 min after an acute use of e-cig (n = 9 EVA study participants). (B) Relative change (from baseline) of urinary 23HPMA at 110, 155, and 200 min after an acute exposure to e-cig-derived aerosols (n = 9 EVA study participants). (C) Urinary levels (ng/mg creatinine) of 23HPMA at 0, 20, 40, 60, 80, 120, and 180 min after an acute use of e-cig or combustible cigarettes (cig) (n = 5 RATS study subjects per group). Values = mean ± SEM. *p < 0.05 vs T1 (0 min) baseline.

expected, neither nicotine nor nicotine metabolites were detected in the urine of mice exposed to HEPA-filtered air or to PG:VG-derived aerosols (Figure 1A).

To further characterize e-cigarette exposures, we screened for metabolites of volatile organic compounds (VOCs) in murine urine in both the first 3 h and the 3−18 h post-exposures. Mice exposed to PG:VG-derived aerosol (6 h) excreted significantly elevated concentrations of the acrolein metabolite (3HPMA; 10×) over basal levels of filtered air-exposed mice (Figure 2A and Figure S2). Similarly, mice exposed to PG:VG-derived aerosol (6 h) excreted significantly elevated concentrations of the glycidol metabolite (23HPMA; 4.5×) over basal levels of filtered air-exposed mice (Figure 2B). Mice exposed to JUUL Virginia Tobacco (JUUL-V)-derived aerosol (6 h) excreted significantly elevated concentrations of the acrolein metabolite (3HPMA; 10×) over basal levels of HEPA filtered air-exposed mice (Figure 2C and Figure S2). Similarly, mice exposed to JUUL-V-derived aerosol (6 h)

https://doi.org/10.1021/acs.chemrestox.1c00328
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excreted significantly elevated concentrations of the glycidol metabolite (23HPMA; 2×) over basal levels of HEPA filtered air-exposed mice (Figure 2D). Mice exposed to JUUL Menthol (JUUL-M)-derived aerosol (6 h) excreted slightly elevated concentrations of the acrolein metabolite (3HPMA) compared with basal levels of HEPA filtered air-exposed mice (Figure 2E and Figure S2). However, mice exposed to JUUL-M-derived aerosol (6 h) excreted significantly elevated concentrations of the glycidol metabolite (23HPMA; 2×) over basal levels of filtered air-exposed mice (Figure 2F). Because of obvious differences in urine nicotine concentrations across products, we also normalized the urine 3HPMA concentration (ng/mL) to the urine total nicotine equivalents (TNE; sum of levels of nicotine, cotinine, and *trans*-3-hydroxycotinine; nmol/mL) (Table 1). After adjusting for TNE, it was clear that MCS had elevated acrolein generation relative to JUUL e-liquids (i.e., 3HPMA/TNE: MCS > JUUL, with JUUL-Mango > JUUL-M, and JUUL-Mango = JUUL-V) (Table 1). However, there were no differences in the concentration of excreted acetate and formate in the urine of mice exposed to filtered air-, PG:VG-, or JUUL e-liquid-derived aerosols at any time point post exposure (Figure S3).

Based on these data, we next asked whether 3HPMA and 23HPMA were derived from a single component of the e-cigarette fluids. Using $^{13}C_3$-labeled VG and UPLC-QTOF MS, we collected the urine of PG:$^{13}C_3$-VG-exposed mice and measured $^{13}C_3$ enrichment in 3HPMA (Figure 3Ai) and in 23HPMA (Figure 3Bi). In fact, there were definitive enrichments and comparable degrees both of $^{13}C_3$-3HPMA (Figure 3Aii) and of $^{13}C_3$-23HPMA (Figure 3Bii) in the first 3 h urine collection post exposure (but not for the 3−18 h post-exposure collection). These data clearly indicate that 3HPMA and 23HPMA were likely metabolic products of acrolein and glycidol formed during VG thermal degradation.

3.2. Human Study. To validate our murine study, we investigated the presence of 3HPMA and 23HPMA in the urine of e-cigarette and combustible cigarette users. Surprisingly, we did not observe an increase in absolute concentration or a relative change of 3HPMA in the urine of e-cigarette users at 110, 155, and 200 min after use (Figure 4A,B). In the second trial, we compared the urinary excretion of 3HPMA using five subjects after the use of an e-cigarette or a combustible cigarette. Although e-cigarette use did not increase 3HPMA excretion, the use of combustible cigarette did increase 3HPMA excretion in urine at 40 and 80 min post use (Figure 4C). Surprisingly, the use of e-cigarettes did increase both the absolute concentration and relative change (approx. 10%) of 23HPMA in the urine of e-cigarette users at 155 min after exposure (Figure 5A,B). In a second trial, we compared the urinary excretion of 23HPMA after e-cigarette or combustible cigarette use. Intriguingly, e-cigarette use appeared to increase 23HPMA urinary excretion at 120 min post use, whereas the use of combustible cigarette did not increase 23HPMA excretion in urine at any time post use (Figure 5C). These preliminary data provide evidence that e-cigarette use in humans may be associated with increased urinary excretion of 23HPMA but not necessarily of 3HPMA.

4. DISCUSSION

Findings of our current study provide further overall evidence that VG—a solvent constituent of all e-liquids—thermally degrades to form toxic compounds including acrolein and glycidol and to increase their respective metabolites in the urine. We present three lines of evidence that support this conclusion: (1) we detect increased levels of acrolein and glycidol metabolites 3HPMA and 23HPMA in the urine of mice exposed to PG:VG- and JUUL e-liquid-derived aerosols; (2) both urinary metabolites—3HPMA and 23HPMA—are enriched in $^{13}C_3$ after mice are exposed to PG:$^{13}C_3$-VG-derived aerosol; and (3) following a brief use, human users of electronic cigarettes have elevated levels of urinary 23HPMA (but not of urinary 3HPMA). The last observation suggests that urinary 23HPMA may be a relatively specific biomarker of e-cigarette use, but further validation will be required. In our controlled human trial of subjects using both e-cigarettes and combustible cigarettes, we find urinary 23HPMA increases in e-cigarette users (at 115 min post exposure) but not in those subjects smoking combustible cigarettes. Supporting this specificity, we found that the urinary levels of 3HPMA increase after combustible cigarette use but not after e-cigarette use.

Our study has many strengths. We have used state-of-the-art mass spectrometry to quantify low levels of urinary VOC metabolites and normalized these levels to creatinine to account for urine concentration/dilution. Our mass spectrometry methodology is adopted from current CDC methods, and the levels measured using our method are consistent with published ranges for these metabolites.[30] Isotopologue analyses of ^{13}C-containing metabolites are via UPLC-QTOF-MS that provides high specificity mass charge (m/z) identification. This approach not only provides validity for non-isotopic identification of 3HPMA and 23HPMA metabolites in urine, but it also provides further evidence linking their formation with the thermal degradation of VG by dehydration into the toxic compounds, acrolein and glycidol. Although thermal degradation of VG is known to generate acrolein along with many other carbonyls,[18,19,25] the metabolites of aldehydes have not been linked experimentally before with the thermal degradation occurring during the use of e-cigarettes. Landmesser *et al.* recently show that combined ^{13}C-PG and ^{13}C-VG in e-liquids lead to ^{13}C-enriched metabolites, yet their results preclude definitive assignment of each ^{13}C-enriched metabolite to either PG or VG degradation.[40] In contrast, our results show that 3HPMA and 23HPMA metabolites are both derived directly from the degradation of VG alone.

Our customized e-cig platform (bluPlus battery coupled with a Mistic bridge cartomizer) is a low-power "cigalike" (<8 W) used under realistic e-cigarette use conditions (91.1 mL puff, 4 s puff, 2 puffs/min),[13] although these are not CORESTA-recommended conditions (55 mL puff, 3 s puff, 2 puffs/min). Thus, the formation of these compounds is not the result of an extreme, high-power setting or "dry puffing" conditions or even the use of a single PG:VG ratio (i.e., we used both 30:70 and 50:50 v:v ratios). Moreover, similar to the dilution of e-cig aerosols in humans using e-cigarettes, the 91.1 mL puff is rapidly diluted >50× (e.g., in a 5 L whole-body exposure chamber similar to lung volume), making our exposures in mice akin to expected "real world" human exposures.

In our experiments, e-cig topography and exposure conditions are kept constant and only the e-liquid is changed. Thus, it is somewhat surprising that exposure of mice to JUUL Menthol e-liquid-derived aerosol significantly increases urinary 23HPMA excretion yet only modestly increases urinary 3HPMA excretion. Regardless of the mechanism underlying these effects of JUUL Menthol e-liquid-derived aerosol

https://doi.org/10.1021/acs.chemrestox.1c00328
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exposure in mice, these results further support the relative utility of 23HPMA as a biomarker of e-cigarette use, whereas 3HPMA production appears more variable (of course, 3HPMA is a robust biomarker of exposure to combustible cigarette smoke[41]). Finally, for our studies, we used both male and female mice as well as human subjects of both sexes, and thus, these results likely are not a result of a sex-specific pathway, but these results are likely generalizable to both sexes.

4.1. Limitations. Our current study has a few limitations. Although we show that 23HPMA is consistently elevated in the urine of mice exposed to PG:VG- and JUUL e-liquid-derived aerosols, the same is not true for our two human studies. However, our human data are taken from a random sample of two relatively small yet controlled panel studies and likely represent the "tip of the iceberg" in that the e-cigarette product landscape cannot be fully represented in any single study. Yet even with our small sample size, we provide a distinct signal that needs to be examined in more depth in a larger human cohort with well-defined e-cig product use and patterns to examine the generalizability of this observation. For example, it may be that certain types of e-cigarettes are more likely to generate more 23HPMA (or 3HPMA *vice versa*) than others based on the PG:VG ratio, power settings, and user topography—none of which were controlled in our present study. Nonetheless, our murine study provides more robust measures of 3HPMA and 23HPMA levels than in our human study that included both infrequent and frequent e-cig users. There are two explanations for this: (1) mice are exposed to aerosols for 6 h, and (2) all murine urine is collected for up to 18 h post exposure. These conditions are, however, necessary because mice have higher background urine levels of both 3HPMA and 23HPMA than humans (i.e., μg/mg creatinine vs ng/mg creatinine) that need to be elevated further to detect significant changes.[17] We did not measure the formation of ^{13}C-parent compounds in the e-cigarette aerosol as Landmesser *et al.* did wherein they show abundant formation of ^{13}C-acetaldehyde, ^{13}C-formaldehyde, and ^{13}C-acrolein yet not ^{13}C-glycidol.[40] The lack of detection of ^{13}C-glycidol in aerosols may result from it being potentially less stable in the presence of acids and metal catalysts.[22] The high concordance between abundant levels of urinary 23HPMA in both e-cig aerosol-exposed mice and human e-cig users in our current study may be a consequence of the generally lower temperatures reached in e-cig devices (<300 °C) than in combustible cigarettes (up to 900 °C). These lower temperature conditions produce less thermal degradation (dehydration) of glycidol into acrolein, and this idea is consistent with the scenario that generation of acrolein from VG proceeds through glycidol formation and is exponentially related to temperature.[18]

In any case, despite the abundance of saturated aldehydes (formaldehyde and acetaldehyde) in PG:VG-derived aerosols, urinary formate and acetate, current biomarkers of their exposure, remain inadequate even under our exposure conditions (6 h to either PG:VG- or JUUL e-liquid-derived aerosols) as shown in Figure S3 and as observed previously.[42] Both urinary formate and acetate levels in rodents are confounded further due to overnight increases presumably due to feeding.[43] Recently, Landmesser *et al.* used ^{13}C-PG and ^{13}C-VG to detect sulfur-containing thiazolidine carboxylic acid and thiazolidine carbonyl glycine metabolites of formaldehyde in urine following inhalation exposure to cigarette smoke or to e-cig aerosols, indicating a potential biomarker of formaldehyde exposure, while a similar biomarker of acetaldehyde inhalation exposure is still needed.[20,40]

5. CONCLUSIONS

Our data provide further evidence for the formation of toxic compounds (glycidol and acrolein) in e-cigarette aerosols and support a hypothesis that the glycidol metabolite 23HPMA may be useful as a relatively specific biomarker of e-cigarette use. Moreover, as cardiopulmonary disease risk appears as a continuum of exposure to acrolein, and as both PG:VG- and JUUL e-liquid-derived aerosols contain acrolein, there is increasing concern that users of e-cigarettes, independent of nicotine or flavorings, may increase cardiopulmonary disease risk. Similarly, as glycidol is also a toxic compound, perhaps product standards should be developed to reduce the levels of acrolein and glycidol generated in e-cigarette aerosols to a level below that which can induce acute and/or chronic cardiopulmonary harm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.1c00328.

Figure S1: images of the electronic cigalike platform used to generate PG:VG- and JUUL e-liquid-derived aerosols; Figure S2: graphic of urinary excretion kinetics of acrolein metabolite in PG:VG- and JUUL e-liquid-derived aerosol-exposed mice; Figure S3: graphic of urinary excretion kinetics of metabolites of acetaldehyde (acetate) and formaldehyde (formate) in JUUL e-liquid-derived aerosol-exposed mice (PDF)

Accession Codes

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author Contributions

P.L., R.K., L.J., J.L., and D.J.C. planned and conducted experiments, generated, analyzed, and interpreted data, and wrote the manuscript. W.T. conducted experiments; T.K. and W.T. generated data and edited the manuscript. D.R. performed statistical analyses and edited the manuscript. A.B. and S.S. interpreted data and edited the manuscript. D.J.C. is the guarantor of this work. As such, he had full access to all data and takes responsibility for the integrity and accuracy of the data.

Funding

This work was supported by the National Institutes of Health (ES023716, GM127607, HL122676, HL149351, U54HL120106, T32ES011564, and S10OD026840) and the Jewish Heritage Fund for Excellence (JHFE).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the University of Louisville Diabetes and Obesity Center Animal Core staff for technical support.

■ ABBREVIATIONS

3HPMA, 3-hydroxypropylmercapturic acid; 23HPMA, 2,3-dihydroxypropylmercapturic acid; CVD, cardiovascular disease; e-cig, electronic cigarette; MCS, mainstream cigarette smoke; PG, propylene glycol; SHS, secondhand smoke; TSP, total suspended particulate; VG, vegetable glycerin; VOCs, volatile organic compounds

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