

Short-Term E-Cigarette Exposure Increases the Risk of Thrombogenesis and Enhances Platelet Function in Mice

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Background—Cardiovascular disease is the main cause of death in the United States, with smoking being the primary preventable cause of premature death, and thrombosis being the main mechanism of cardiovascular mortality in smokers. Due to the perception that electronic/e-cigarettes are “safer/less harmful” than conventional cigarettes, their usage—among a variety of ages—has increased tremendously during the past decade. Notably, there are limited studies regarding the negative effects of e-cigarettes on the cardiovascular system, which is also the subject of significant debate.

Methods and Results—We employed a passive e-Vape™ vapor inhalation system and developed an in vivo whole-body e-cigarette mouse exposure protocol that mimics real-life human exposure scenarios/conditions and investigated the effects of e-cigarettes and clean air on platelet function and thrombogenesis. Our results show that platelets from e-cigarette-exposed mice are hyperactive, with enhanced aggregation, dense and α granule secretion, activation of the α IIb β 3 integrin, phosphatidylserine expression, and Akt and ERK activation, when compared with clean air-exposed platelets. E-cigarette-exposed platelets were also found to be resistant to inhibition by prostacyclin, relative to clean air. Furthermore, the e-cigarette-exposed mice exhibited a shortened thrombosis occlusion and bleeding times.

Conclusions—Taken together, our data demonstrate for the first time that e-cigarettes alter physiological hemostasis and increase the risk of thrombotic events. This is attributable, at least in part, to the hyperactive state of platelets. Thus, the negative health consequences of e-cigarette exposure should not be underestimated and warrant further investigation. (*J Am Heart Assoc.* 2018;7:e009264. DOI: 10.1161/JAHA.118.009264.)

Key Words: cardiovascular disease • e-cigarettes • electronic nicotine delivery systems • e-vaping • platelet • thrombosis

Cardiovascular disease (CVD) is the single largest cause of death in the United States, with smoking being responsible for 1 of every 3 deaths linked to CVD.¹ Thus, over the past few years, the introduction of electronic cigarettes (e-cigarettes), also called electronic nicotine delivery systems, was considered rather transformational, with a wide variety of individuals of all ages switching to them and/or commencing on their use. Indeed, it is alarming that the highest increase in e-cigarette usage is among youth, which could be attributable

to their curiosity, the appealing nature of e-liquids, and the aggressive advertisement.^{2,3} The increased popularity of e-cigarettes can also be attributed to the belief that they are less harmful compared with traditional tobacco smoking. Because e-cigarettes were thought/claimed to generate less noxious materials/toxicants,^{4–6} ultimately at the time, they were embraced as a safer alternative to tobacco smoke. Nonetheless, the negative consequences of e-cigarettes, including their impact on the cardiovascular system,⁷ remain a subject of debate, especially in light of the fact they are not emission-free devices. To this end, e-cigarettes in fact share some of the toxicant profile of conventional cigarette smoking and may expose users to similar health risks.^{8–10}

In terms of their impact on the cardiovascular system, thus far there have been a limited number of studies examining the effects of e-cigarettes on the development of CVD. Furthermore, some of these studies were contradictory, with some reporting no cardiovascular harm,^{11,12} whereas others found e-cigarettes to be harmful.^{13,14} In support of the latter notion, several studies have shown that e-cigarettes are associated with an increase in blood pressure, aortic stiffness, endothelial dysfunction, and oxidative stress.^{6,13–15} Interestingly, a

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Clinical Perspective

What Is New?

- This is the first study to demonstrate that, in an animal model, whole body exposure to e-cigarettes enhances platelet activation and increases the risk of thrombogenesis.

What Are the Clinical Implications?

- Based on our observations, we expect that clinicians will help in educating the public and their patients regarding the potentially negative cardiovascular health effects of e-cigarettes and the evidence that they may not be as safe as currently perceived.

recent study reported that e-cigarette–induced impairment of cardiovascular function in mice was similar to that triggered by conventional smoking, in which a comparable increase in aortic stiffness was observed.¹⁴

To our knowledge, studies on the effects of e-cigarettes on the genesis of thrombosis-dependent cardiovascular disorders have been limited to only 1 thus far. To this end, albeit associated with limitation, this study reported that exposure of platelets to e-cigarette extracts *in vitro* enhanced platelet activation¹⁶ (eg, aggregation and adhesion). Consequently, findings from this study suggested that e-cigarettes may potentially increase the risk of thrombosis.¹⁶ However, this study neither mimicked real-life conditions (eg, employed e-cigarette extracts) nor used an *in vivo* whole-body exposure model to investigate the effects of e-cigarettes on platelets. It is noteworthy that thrombosis is the main mechanism of cardiovascular mortality in smokers¹⁷; smoking causes a prothrombotic state through altering fibrinolytic and thrombotic factors^{17,18}; smokers' isolated platelets exhibited increased aggregation^{19,20}; and exposure to cigarette smoke alters the dynamics of clot formation, making them more resistant to thrombolysis as compared with clots of non-smokers.^{21–23} However, whether e-cigarettes exert similar effects is yet to be determined and warrants investigation.

Based on the aforementioned considerations, the current study investigated the mechanistic impact of e-cigarettes on platelet function and thrombogenesis in mice by employing a novel whole-body/*in vivo* model that resembles real-life e-cigarette exposure scenarios. Our findings revealed that short-term e-cigarette exposure causes a hyperactive state of platelets, which are also resistant to inhibition by prostacyclin, and that it elevates the risk of thrombosis, and enhances hemostasis. On the other hand, leukocyte activation was found to be no different between e-cigarettes and clean air. Collectively, these data do support the notion that e-cigarette exposure is detrimental to cardiovascular health and is an important player in thrombosis-related disease states.

Methods and Materials

Reagents and Materials

Absolute Zero e-liquid (18 mg nicotine, 30% propylene glycol, 70% vegetable glycerin with a menthol flavor) was obtained from The Vapor Chef (Bristol, PA). ADP, prostacyclin/prostaglandin I₂ (PGI₂), cotinine methanol solution, and cotinine-D3 standard were purchased from Sigma Aldrich (St. Louis, MO). U46619 was purchased from Abcam (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin and FITC-conjugated Annexin V were purchased from Cell Signaling Technology, Inc (Danvers, MA). The JON/A antibody was obtained from Emfret analytics (Würzburg, Germany). The phycoerythrin-conjugated anti-CD69 and FITC-conjugated anti-CD45 were obtained from BD Biosciences (San Jose, CA). Stir bars and other disposables were purchased from Chrono-Log Corporation (Havertown, PA). Kinetex 1.7 μm EVO C18 100 A-LC Column 100×2.1 mm, Security Guard ULTRA holder for UHPLC Columns 2.1 to 6.4 mm, and SecurityGuard ULTRA cartridges for EVO-C18-UHPLC were purchased from Phenomenex Inc (Torrance, CA). Other reagents were of analytical grade.

Animals

C57BL/6 10J (10-week-old male) mice (referred to hereafter as C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, ME), and were housed in groups of 1 to 4 at 24°C, under 12/12 light/dark cycles, with access to water and food *ad libitum*. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at El Paso.

Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Because there are no specific study materials *per se*, requests for data, the vaping protocol, and/or liquid chromatography–mass spectrometry methodology will be honored on a case-by-case basis.

E-Cigarette Exposure Protocol

C57BL/6 mice were exposed over 2 sessions to a total of 200 puffs per day, and the exposure lasted for 5 days/ 1 week. A 15-minute break between the 2 exposure sessions was given so that the animals could access fresh air, food, and water. The puff duration was 3 seconds, puff interval 1 minute, and the puff volume was 50 mL, all of which mimics real-life exposure scenarios.^{24–33} As for the controls or control mice,

they were matched in terms of age and sex (ie, 10-week-old males), and were exposed to clean air. The clean air group was not given food and water during the exposure times. Experiments were performed 1 hour after the last exposure session.

Aerosol (e-vape) was generated using a 4-chamber bench-top passive e-Vape™ vapor inhalation system, for mice, from the La Jolla Alcohol Research Inc (La Jolla, CA). As for the e-cigarette device utilized for the inhalation system, we used the SMOK TFV4 Mini Tanks, and the settings were 5 V for voltage, 0.4 ohm for resistance, and 1 L/min for flow rate, all of which resemble common e-cigarette devices.^{34,35} The e-liquid selected for our experiments was one of the top sellers,³⁶ namely, the Vapor Chief (18 mg/mL nicotine content). It contains propylene glycol/vegetable glycerin at a 30/70 ratio, with a menthol flavor.

Liquid Chromatography–Tandem Mass Spectrometry Analysis of Cotinine

The plasma concentrations of cotinine, the major metabolite of nicotine was determined using liquid chromatography–tandem mass spectrometry, as described before,³⁷ with some modifications. Cotinine was extracted from 20 μ L of plasma from animals exposed or not to e-cigarette smoke by the addition of 160 μ L acetonitrile (1:8, v/v). Prior to organic extraction, plasma samples were spiked with deuterated cotinine-*d*3 (20 ng/mL, internal standard). Precipitated proteins in the samples were removed after homogenization for 1 minute in a Vortex-Genie 2 (Scientific Industries, Inc), followed by centrifugation (25 minutes, 15 100g, at 4°C), using a 4515D Eppendorf microcentrifuge. Five microliters of the resulting supernatant, containing nicotine and cotinine (and other metabolites), was analyzed by liquid chromatography–tandem mass spectrometry. Metabolites were separated and analyzed by reversed-phase chromatography using a Phenomenex (Kinetex 1.7 μ m, EVO C18, 100Å, 100×2.1 mm) column equipped with an additional guard column (SecurityGuard ULTRA cartridge, EVO C18), in a Dionex UHPLC Ultimate 3000 RS (Thermo Fisher Scientific) coupled to a TSQ Endura Triple Quadrupole Mass Spectrometer (TSQ Endura MS) (Thermo Fisher Scientific). Metabolite separation was achieved in a gradient of solvent A (H₂O, 0.1% formic acid) and solvent B (acetonitrile: methanol, 3:1, v/v) with a constant flow rate of 0.5 mL/min. The column was equilibrated with 10% solvent B and was maintained for 0.5 minute after starting the run. The elution consisted of a 2-minute gradient up to 90% solvent B, which was held for additional 2.5 minutes. The total duration of the reversed-phase chromatography was 5 minutes. After each sample run, a blank injection (90% solvent B) was used for reequilibration of the column with 90%

solvent B, starting the blank injection for 0.5 minutes, then a sharp decrease to 10% solvent B over 0.5 minutes. The starting conditions of 10% solvent B was then maintained for 2 minutes, for a total runtime of 3 minutes. All solvents used above were liquid chromatography–mass spectrometry grade from Thermo Fisher Scientific. Quantification of cotinine was achieved by highly sensitive and specific single-reaction monitoring approach, using product-ions *m/z* 80.45 and 146.20, for cotinine and cotinine-*d*3, respectively. The TSQ Endura MS was equipped with a heated electrospray ionization source and set to the following parameters: positive ion voltage of 5700 V; sheath, auxiliary, and sweep gas of 10, 7, and 5 arbitrary units, respectively; and ion-transfer tube and vaporizer temperature of 350°C and 300°C, respectively.

Tail Bleeding Time Assay

C57BL/6 mice were exposed as discussed above, and the tail bleeding assay was performed as we described before.^{38–40} Briefly, mice were anesthetized and placed on a 37°C homeothermic blanket, before the tail was transected 5 mm from the tip using a sterile scalpel. After transection, the tail was immediately immersed in saline (37°C constant temperature), and the time to bleeding cessation was measured. Bleeding stoppage was not considered complete until bleeding had stopped for 1 minute. For statistical analysis purposes, if bleeding did not stop in 15 minutes, the experiment was halted to limit blood loss from the mice, and 15 minutes was considered the cutoff bleeding time.

In Vivo Ferric Chloride Carotid Artery Injury–Induced Thrombosis Model

C57BL/6 mice were exposed as discussed above, and the assay was performed as we described before.^{38–40} Briefly, mice were anesthetized with Avertin (2.5%), and the left carotid artery was exposed and cleaned with normal saline (37°C), before baseline carotid artery blood flow was measured with Transonic Micro-Flowprobe (Transonic Systems Inc, Ithaca, NY). After stabilizing blood flow, 7.5% ferric chloride was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 minutes. Blood flow was continuously monitored for 20 minutes or until blood flow reached stable occlusion (no blood flow for 2 minutes). Data were recorded, and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with ferric chloride). An occlusion time of 20 minutes was considered as the cutoff time for statistical analysis.

Platelet Count

Platelet count was performed on whole blood obtained from the exposed mice, using a HEMAVET[®] 950FS Multi-species Hematology System from Erba[®] Diagnostics (Miami Lakes, FL).

Murine Platelet-Rich Plasma Preparation

Clean air- or e-cigarette-exposed mice were anesthetized, and blood was collected from the heart. Coagulation was inhibited by 0.38% sodium citrate solution (Fisher Scientific, Hampton, NH). Blood was centrifuged (237*g* for 15 minutes) at room temperature, and the platelet-rich plasma was then collected. Platelets were counted with the HEMAVET[®] 950FS Multi-species Hematology System, and their count adjusted to 7×10^7 platelets/mL before each experiment.

Washed Platelet Preparation

Washed platelets were prepared as described previously.^{38,41,42} Mouse blood was collected as discussed above and mixed with phosphate-buffered saline, pH 7.4, and was incubated with PGI₂ (10 ng/mL; 5 minutes), followed by centrifugation at 237*g* for 10 minutes at room temperature. Platelet-rich plasma was recovered, and platelets were pelleted at 483*×g* for 10 minutes at room temperature. The pellets were resuspended in HEPES/Tyrode buffer (20 mmol/L HEPES/KOH, pH 6.5, 128 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl₂, 0.4 mmol/L NaH₂PO₄, 12 mmol/L NaHCO₃, 5 mmol/L D-glucose) supplemented with 1 mmol/L EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI₂. Platelets were then washed and resuspended in HEPES/Tyrodex (pH 7.4) without EGTA, apyrase, or PGI₂. Platelets were counted using the HEMAVET[®] 950FS Multi-species Hematology System and adjusted to the indicated concentrations.

In Vitro Platelet Aggregation

Platelet-rich plasma from clean air- or e-cigarette-exposed mice was activated with ADP (0.5–2.5 μmol/L) and U46619 (2 μmol/L). Platelet aggregation was measured by the turbidometric method using model 700 aggregometer (Chrono-Log Corporation). We also performed these aggregation experiments with ADP (2.5 μmol/L) and U46619 (2 μmol/L) in the presence (3 minutes incubation) or absence of 5 nmol/L prostacyclin (PGI₂). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups (ie, at least 5 mice each) that were exposed to either e-cigarettes or clean air.

ATP Release

Platelet-rich plasma was prepared as described above (250 μL; 7×10^7 /mL) before being placed into siliconized cuvettes and stirred for 5 minutes at 37°C. The luciferase substrate/luciferase mixture (12.5 μL, Chrono-Log) was then added, followed by the addition of the agonists ADP (2.5 μmol/L) and U46619 (2 μmol/L).

Flow Cytometric Analysis

Flow cytometric analysis was performed as we described previously.³⁸ Briefly, washed platelets (2×10^7 /mL) from clean air- or e-cigarette-exposed mice were stimulated with ADP (10 μmol/L) and U46619 (5 μmol/L) for 5 minutes. Platelets were then fixed with 2% formaldehyde for 30 minutes at room temperature and incubated with FITC-conjugated CD62P (P-selectin), Annexin V or phycoerythrin-conjugated rat anti-mouse integrin α_{IIb}β₃ (active form) JON/A antibodies at room temperature for 30 minutes in the dark. The platelet (10^5 platelets/100 μL) fluorescent intensities were measured using a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). Results were analyzed using CFlow Plus (BD Biosciences). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups (at least 5 mice each) that were exposed to either clean air or e-cigarettes.

Leukocyte Activation

The activation state of leukocytes was examined using flow cytometry, as described before.^{43,44} Briefly, whole blood from e-cigarette- and clean air-exposed mice (unstimulated samples) was incubated with either FITC-conjugated anti-CD45 or phycoerythrin-conjugated anti-CD69 for 20 minutes at room temperature in the dark. The reaction was stopped by adding BD FACS™ lysing solution (1:10 in phosphate-buffered saline), and the samples were kept at room temperature for 15 minutes in the dark. Next, the samples were washed with 1X phosphate-buffered saline, before being fixed with 1% formaldehyde for 15 minutes. Samples were transferred to FACS tubes, and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences).

Immunoblotting

Immunoblot was carried out as described before.⁴² Briefly, clean air- or e-cigarette-exposed washed platelets were stimulated with ADP (2.5 μmol/L) or U46619 (5 μmol/L) for 3 minutes followed by lysis with 1X sample buffer. Next, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P

PVDF membranes (Bio-Rad, Hercules, CA). Membranes were then probed with the primary antibodies (ERK, pERK, Akt, and pAkt) and visualized with horseradish peroxidase–labeled antirabbit or antimouse immunoglobulin G as required. The antibody binding was detected using enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were obtained with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA), and quantified with Image Lab software Version 4.1 (Bio-Rad).

Statistical Analysis

All experiments were performed at least 3 times with blood pooled from 3 groups of at least 5 mice each time, as applicable. Data analysis was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean±standard error of mean. The Mann-Whitney test (non-parametric test) was used for the evaluation of differences in mean occlusion and bleeding times, whereas the t test and/or 1-way ANOVA with Tukey's multiple comparisons test as post hoc were used for the analysis of the flow cytometry data, as applicable (per number of groups). Notably, no differences were observed with regard to significance when these data were analyzed using the Mann-Whitney test/ANOVA, in comparison to the t test. Significance was accepted at $P<0.05$, unless stated otherwise.

Results

E-Cigarette Exposure Systematically Delivers Nicotine, in Mice

Cotinine is the major metabolite of nicotine, and serves as a marker of systematic nicotine “delivery.”⁴⁵ Therefore, in order to confirm that our e-cigarette whole-body exposure did result in the systemic delivery of nicotine, plasma cotinine levels were measured using liquid chromatography–tandem mass spectrometry. Our results revealed that there were significant levels of cotinine in the mice exposed to e-cigarettes, whereas it was not detectable in the clean air control mice. Importantly, the measured mean plasma levels (ie, average of 53.97 ± 3.61 ng/mL; Figure 1) were in fact comparable to those observed in humans and other animal e-cigarette exposure models, that is, 30 to 250 ng/mL.^{46–48}

E-Cigarette Exposure Alters Physiological Hemostasis and Thrombosis Development

Although cigarette smoking has been shown to produce effects in vivo, for example, to increase the risk of various disease states, including those involving thrombogenesis,^{49,50} whether e-cigarette exposure produces similar effects or not

remains to be determined. Thus, we first evaluated the effect of e-cigarette exposure on the hemostasis response by conducting the tail bleeding time assay in live, anesthetized mice. Indeed, it was found that the time needed for cessation of bleeding was significantly shortened in the e-cigarette–exposed mice (average of 52 ± 18 seconds; Figure 2A), compared with those exposed to clean air (average of 585 ± 15 seconds; Figure 2A), suggesting a prothrombotic state. On this basis, we hypothesized that e-cigarette–exposed mice would be more prone to thrombosis. Consequently, we next sought to examine the impact of e-cigarettes on thrombogenesis using the ferric chloride carotid artery injury–induced thrombosis model. Our data showed that the e-cigarette–exposed mice had a shortened occlusion time (average of 248 ± 69 seconds; Figure 2B), compared with the clean air controls (average of 612 ± 67 seconds; Figure 2B). Together, these findings demonstrated for the first time that e-cigarettes do indeed impact (enhance) physiological hemostasis and increase the risk of thrombotic events.

E-Cigarette Exposure Does Not Affect Platelet Count

Platelets play an integral role in maintaining hemostasis, and changes in their counts have been linked to mortality and CVD development.²⁴ Furthermore, while the effect of “smoke” on platelet count remains controversial, with studies showing it to be increased⁵¹ or unaffected,^{52,53} we sought to examine whether e-cigarettes would produce any effects under our experimental conditions. Our data revealed no difference in platelet count between clean air– and e-cigarette–exposed mice, that is, 526 ± 64 versus 571 ± 96 (thousand/ μ L; $P=0.323$), respectively, at least under the present experimental conditions. Thus, these data eliminate abnormal platelet count as a contributing factor to the observed phenotype.

E-Cigarette Exposure Enhances Agonist-Induced Platelet Activation/Aggregation

While our data revealed that e-cigarette exposure modulates hemostasis and thrombus formation, whether it has any impact on platelet function is yet to be investigated. To this end, it was recently shown that treatment with e-cigarette extracts enhances platelet activation¹⁶; whether such effects would manifest under whole-body e-cigarette exposure experimental settings is unknown. Thus, we next sought to investigate the effects of e-cigarette exposure on G-protein–coupled receptor agonist–induced platelet aggregation. It was observed that platelets from e-cigarette–exposed mice exhibited higher aggregation, in comparison with the clean air–exposed controls, in response to several agonists, that is, ADP and U46619 (Figure 3A and 3B). These data suggest that

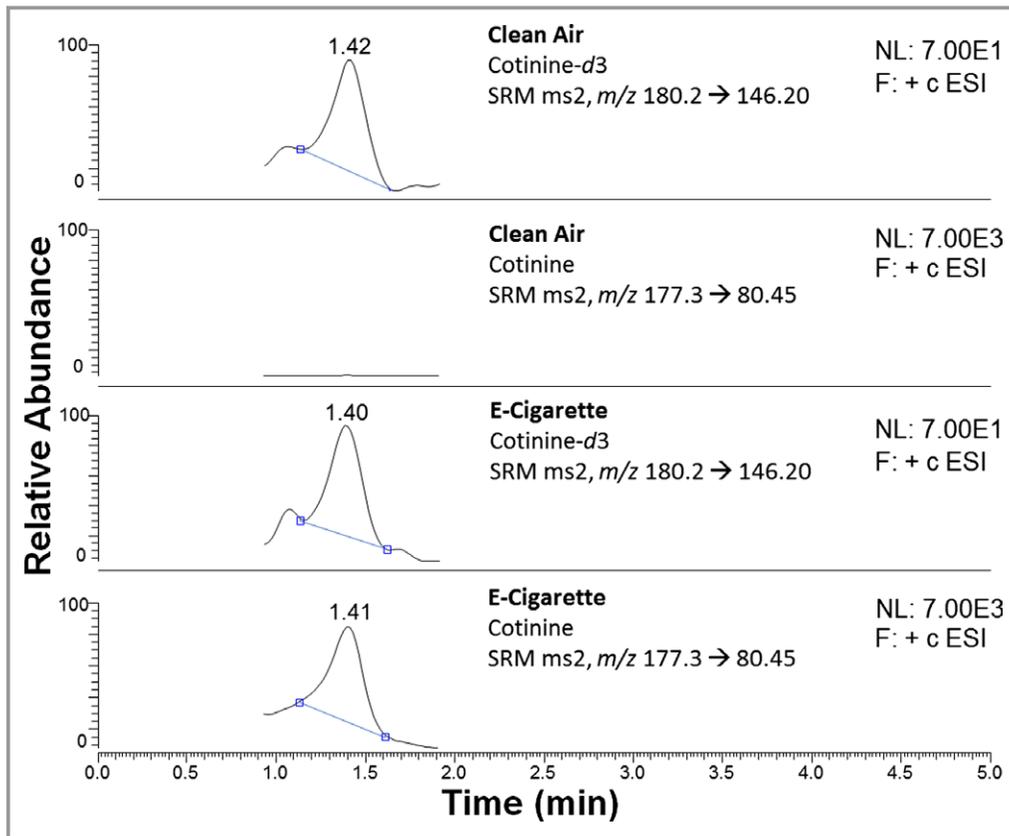


Figure 1. E-cigarette exposure results in systemic delivery of nicotine measured as the marker cotinine. Plasma from clean air or e-cigarette-exposed mice was subjected to liquid chromatography coupled with tandem mass spectrometry, as described in the “Methods” section, and the levels of cotinine were determined. Briefly, plasma samples were spiked with 20 ng/mL cotinine-d3 (internal standard) before organic solvent extraction of nicotine and its major metabolite, cotinine. Metabolites were separated by reversed-phase chromatography/reversed-phase chromatography and detected and quantified by liquid chromatography–tandem mass spectrometry (positive-ion mode) using selected reaction monitoring. Peaks corresponding to major product ions for cotinine-d3 (m/z 146.20) and cotinine (m/z 80.45) were integrated for quantification as indicated. Chromatograms are representative of biological duplicates and technical triplicates. Measurements were performed on several individual animals from at least 3 different exposures.

e-cigarette-exposed platelets are hyperactive, which is consistent with the shortened bleeding and occlusion times observed in these mice.

E-Cigarette Exposure Enhances Agonist-Induced Platelet Secretion

Given the important role of agonist-induced exocytosis (granules release) in amplifying the initial platelet activation events,^{54,55} we examined the impact of e-cigarettes on dense (ATP) and α granule (P-selectin) secretion/release. In accordance with our aggregation data, ATP secretion induced by agonists (ADP and U46619; Figure 4A and 4B), was enhanced in platelets obtained from e-cigarette-exposed mice relative to the clean air controls. Similarly, platelets from mice exposed to e-cigarettes had significantly higher agonist (ADP

and U46619)-triggered expression of P-selectin on their surface (Figure 4C and 4D; the percentage of P-selectin-positive cells induced by ADP was 61.17 ± 5.54 [e-cigarettes] versus 33.33 ± 3.93 [clean air]; $P=0.0014$; whereas that induced by U46619 was 60.33 ± 4.63 [e-cigarettes] versus 33.33 ± 3.28 [clean air]; $P=0.0089$), compared with controls. These findings indicate that whole-body exposure to e-cigarettes upregulates both dense and α granule secretion, supporting the notion that these platelets are indeed hyperactive.

E-cigarette Exposure Enhances Agonist-Induced Integrin $\alpha_{IIb}\beta_3$ Activation

In the next set of experiments, we investigated whether the increased aggregation response observed in the

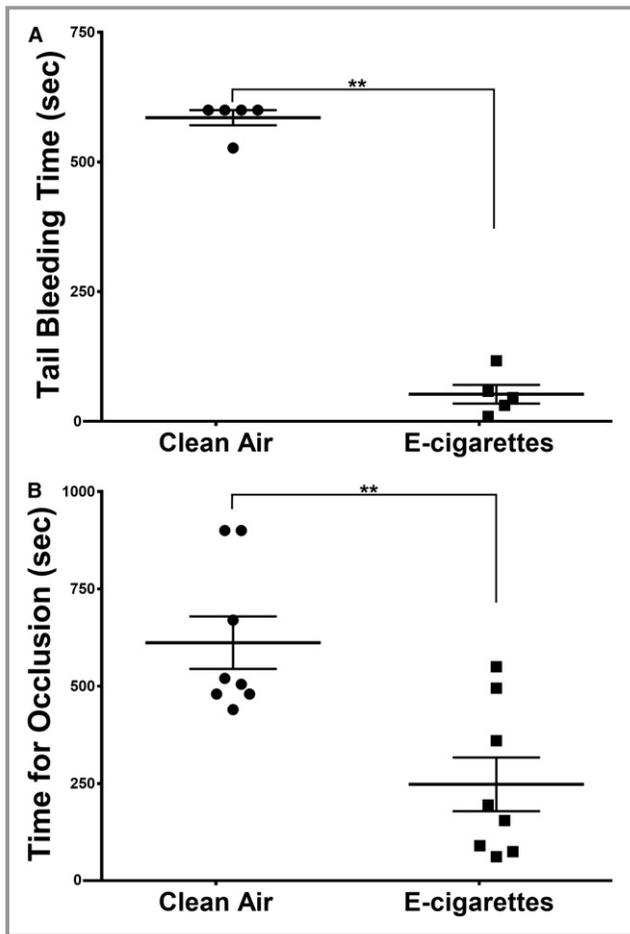


Figure 2. E-cigarette exposure shortens the tail bleeding time and the time for occlusion. A, E-cigarette- and clean air-exposed mice were subjected to the tail bleeding time assay, as described in the “Methods” section. Each point represents the tail bleeding time of a single animal (clean air, n=5; and e-cigarette, n=5; ** $P<0.01$). B, E-cigarette- and clean air-exposed mice were subjected to the ferric chloride-induced thrombosis model, as described in the “Methods” section, and the time for occlusion was measured. Each point represents the occlusion time of a single animal (clean air, n=8; and e-cigarette, n=8; ** $P<0.01$).

e-cigarette-exposed mice is accompanied by a commensurate elevation in integrin GPIIb-IIIa ($\alpha_{IIb}\beta_3$) activation; which is required for platelet aggregation.⁵⁶ We observed that the activation of the $\alpha_{IIb}\beta_3$ integrin is enhanced in e-cigarette-exposed platelets, compared with the clean air exposure, in response to ADP and U46619 (Figure 5A and 5B; the percentage of $\alpha_{IIb}\beta_3$ -positive cells induced by ADP was 76.33 ± 2.35 [e-cigarettes] versus 49 ± 3.46 [clean air]; $P=0.0028$; whereas that induced by U46619 was 85.67 ± 2.33 [e-cigarettes] versus 59.33 ± 3.18 [clean air]; $P=0.0023$). These data are in support/consistent with the enhanced aggregation (and secretion) response in the e-cigarette-exposed platelets.

E-Cigarette Exposure Enhances Agonist-Induced Phosphatidylserine Expression

Phosphatidylserine (PS) exposure on the surface of activated platelets is known to be essential for the assembly of coagulation factor complexes,⁵⁷ and its appearance on the cell surface is associated with several physiologic and pathologic phenomena.^{58,59} Hence, we determined whether e-cigarette exposure modulates the surface levels of phosphatidylserine. Similar to integrin activation, agonist (ADP and U46619)-induced phosphatidylserine expression was higher in the e-cigarette-exposed platelets compared with those from the clean air mice (Figure 6A and 6B).

In summary, our data thus far show that platelets from e-cigarette-exposed mice exhibit enhanced platelet function, namely, aggregation, secretion, integrin activation, and phosphatidylserine exposure, compared with those from the clean air-exposed mice.

E-Cigarette Exposure Enhances Agonist-Induced Akt and ERK Phosphorylation

It was previously shown that phosphorylation of Akt and/or ERK plays a critical role in platelet function,⁶⁰⁻⁶² and supports thrombus formation.^{63,64} Thus, we examined whether e-cigarettes exert any effect on these 2 markers of platelet activation. Indeed, our results revealed that ADP-, and U46619-induced Akt and ERK phosphorylation is enhanced in e-cigarette-exposed platelets, relative to clean air (Figure 7A and 7B, respectively; densitometric analyses are shown in Figure 7C through 7F). These data provide biochemical evidence that Akt and ERK activation contributes to the e-cigarette-induced hyperactive platelet phenotype, in addition to the functional and “disease” data demonstrating that e-cigarettes do modulate platelet function.

E-Cigarette Exposure Renders Platelets Resistant to Prostacyclin Inhibition

It is well known that exposure to traditional tobacco makes platelets more resistant to inhibition by the endothelial cells’ PGI₂. Therefore, we sought to determine whether e-cigarettes would produce similar effects. Indeed, we observed that PGI₂ (5 nmol/L) was not as effective in inhibiting ADP- or U46619-induced aggregation in platelets from e-cigarette-exposed mice, in comparison to those from clean air-exposed mice (Figure 8A and 8B). These data indicate that e-cigarette-exposed platelets are more resistant to inhibition by PGI₂ than are those exposed to clean air.

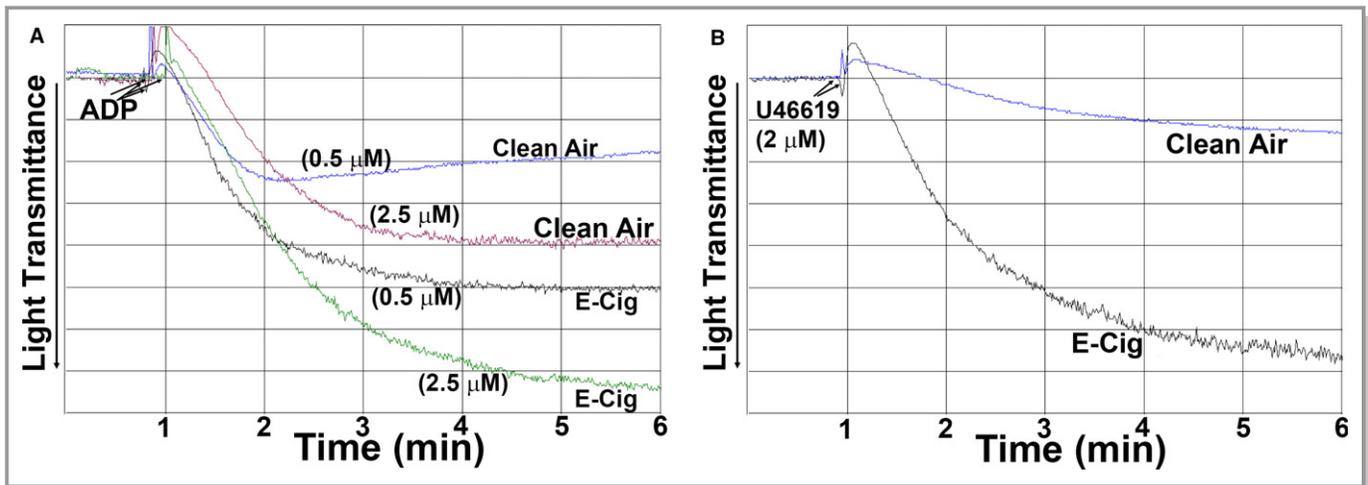


Figure 3. E-cigarette exposure enhances platelet aggregation. Platelets from e-cigarette- and clean air-exposed mice were stimulated with 0.5 $\mu\text{mol/L}$ and 2.5 $\mu\text{mol/L}$ ADP (A), or 2 $\mu\text{mol/L}$ U46619 (B), and their aggregation response was monitored using an aggregometer. Each experiment was repeated 3 times, with blood pooled from at least 5 to 6 mice each time. E-Cig indicates E-Cigarettes.

E-Cigarette Exposure Does Not Affect Leukocyte Activation

While traditional tobacco is known to potentiate leukocyte activation,^{43,44} it remains to be determined if e-cigarettes produce such effects. Interestingly, our results revealed no difference in leukocyte activation between e-cigarettes and the clean air exposures (Figure 9A and 9B); under the present experimental conditions.

Discussion

Cigarette smoking is the primary preventable cause of premature death in the United States,⁶⁵ accounting for more than 18% of annual deaths.⁶⁶ Consequently, and due (at least in part) to the perception of e-cigarettes' "higher safety profile," their use has increased drastically in the past decade.^{9,67-71} Importantly, these devices do emit considerable levels of toxicants, some of which are shared/overlap with tobacco smoking; and thus their harm should not be underestimated.^{6,72} Therefore, in contrast to the well-known deleterious effects of tobacco smoking, and despite the exponential increase in e-cigarette research, their safety profile has not been fully studied in the context of thrombotic disease. This is due in part to serious methodological problems/limitations in the human studies, including study groups (eg, former smokers' versus nonsmokers); use of different e-cigarette devices and e-liquids; and (until recently) lack of validated whole-body exposure (animal) models, in which the aforementioned variables can be controlled to resemble real-life exposure conditions.

Based on these considerations, we utilized an *in vivo* whole-body exposure model and established/designed an

exposure protocol that resembles real-life scenarios. Thus, key markers to be noted of this system are (1) the cotinine levels found in the plasma of our e-cigarette-exposed mice (average of 53.97 ng/mL) overlaps with those observed in established human and animal e-cigarette exposure models⁴⁶⁻⁴⁸ but undetectable in clean air samples, (2) which were obtained/observed using a device and an e-liquid mixture that are widely used by humans^{34-36,73} and (3) under real-life puffing topography experimental conditions.²⁴⁻³³

Notably, our whole-body exposure system, much like active and passive e-cigarette exposure,^{47,74} did increase cotinine (plasma) levels supporting the systematic delivery of nicotine, and further undermining the claim that e-cigarettes are emission-free devices; this finding also casts doubt regarding the notion that e-cigarette are a safer/safe alternative to tobacco. Further support for the validity and the clinical applicability of our animal model derives from literature documenting that mouse studies do "map" very well to humans in the context of tobacco exposure.^{14,75,76} In fact, given the challenges in human studies and the difficulty in drawing conclusions due to the variability in the device and e-liquid products, users' experience, and concomitant exposure to other forms of tobacco, the current model gives us great flexibility in mimicking many aspects of human exposure.

After "validating" our model, we first sought to investigate the impact of e-cigarettes on thrombogenesis, as it is the major cause of tobacco-induced CVD,³⁸ including thrombosis-based diseases.^{17,77-83} Our findings revealed, for the first time, a drastic reduction in the time for carotid artery occlusion, indicating a prothrombotic phenotype, even after only a week of whole-body exposure to e-cigarettes. These data should cast serious doubt concerning the presumed

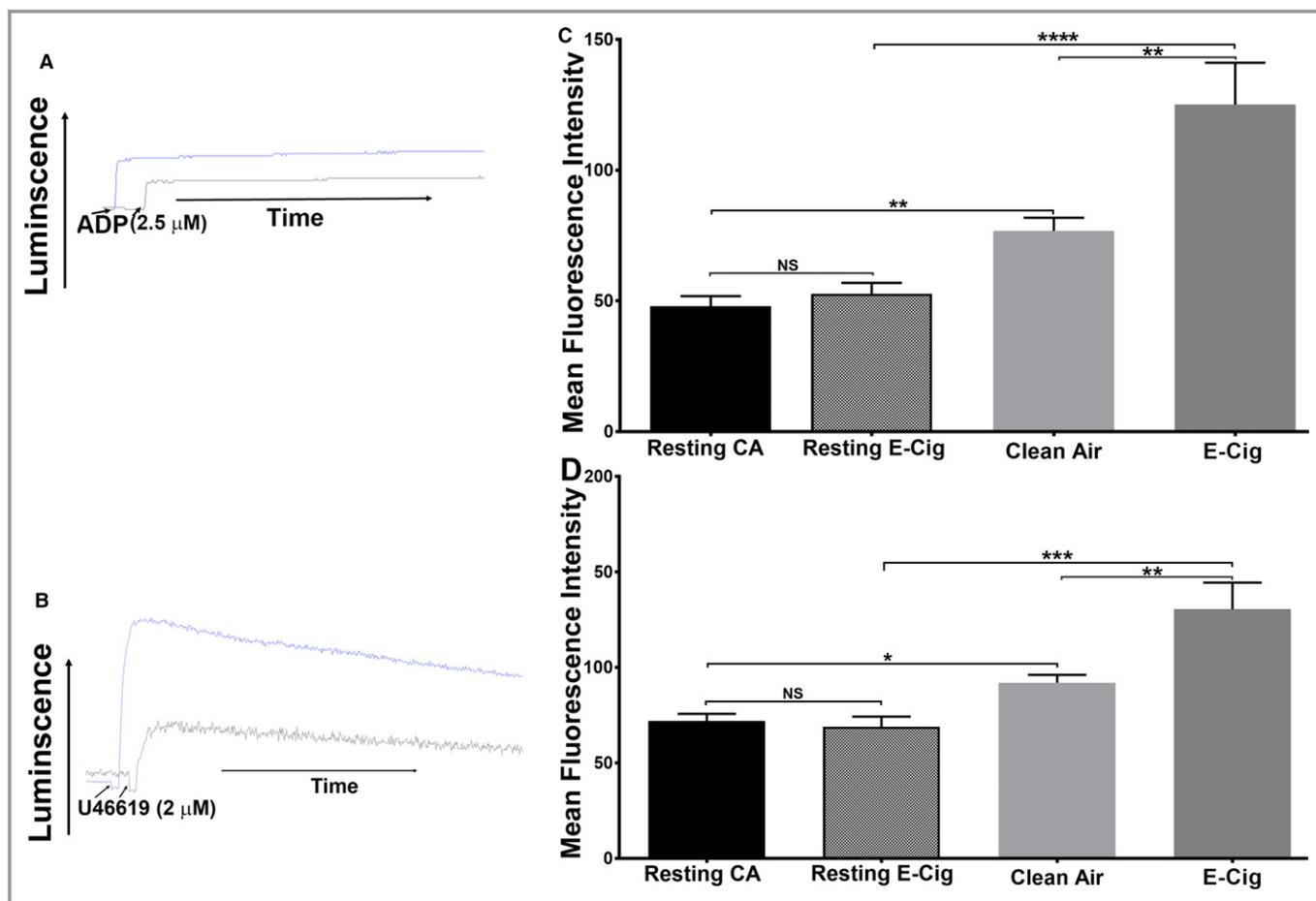


Figure 4. E-cigarette exposure enhances platelet secretion. Platelets from e-cigarette- and clean air-exposed mice were incubated with luciferase luciferin (12.5 μ L), before being stimulated with 2.5 μ mol/L ADP (A) or 2 μ mol/L U46619 (B). ATP release (for dense granules) was detected as luminescence, and measured by a lumi aggregometer. Platelets from e-cigarette- and clean air-exposed mice were washed, before stimulation with 10 μ mol/L ADP (C) or 5 μ mol/L U46619 (D). Platelets were incubated with fluorescein isothiocyanate-conjugated CD62P antibody (for α granules), and the fluorescent intensities were measured by flow cytometry. Average mean fluorescence intensities shown (* P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001; NS, nonsignificant). Each experiment was repeated 3 times, with blood pooled from at least 5 to 6 mice each time. CA indicates clean air; E-Cig, E-Cigarettes.

safety of e-cigarettes, and could be attributed, at least in part, to the high “sensitivity” of the cardiovascular system, and the nonlinear dose-response relationship for toxicity.^{84,85} Thus, even exposure to low levels of harmful constituents “as claimed” with e-cigarettes has the potential to induce pronounced effects.⁸⁶ This notion is consistent with findings that switching to e-cigarettes did not significantly reduce the risk of acute myocardial infarction or stroke.⁸⁷

Consistent with the observed prothrombotic phenotype, evidence exists showing that acute exposure to e-cigarettes in humans, even after 30 minutes of exposure, significantly increased arterial stiffness,¹³ which is a marker for increased thrombosis-dependent CVD.⁸⁸ In addition, an analysis of the National Health Interview Survey database in 2014 revealed that use of e-cigarettes increases the odds of myocardial infarction by 42%.⁸⁹ Furthermore, a case of acute myocardial infarction in a young man was recently

reported after only 1 month of e-cigarette use.⁹⁰ Separate studies showed that e-cigarettes also induce myocardial DNA damage,⁹¹ arterial stiffness¹⁴ and stroke⁹² in mice. While the current data indicate that short-term exposure to e-cigarettes is associated with cardiovascular harm, one can speculate that longer use/exposure to e-cigarette would have even more detrimental effects; this will be the scope of future investigations.

As for the hemostasis response in the e-cigarette-exposed mice, our data showed they possessed a significantly shortened tail bleeding time. This finding is not surprising given the “thrombosis” phenotype we observed and is supported by previous studies by us and others, in which exposure to tobacco and its constituents was found to reduce the tail bleeding time.^{38,93} Of note, our data revealed that e-cigarettes do not modulate/reduce platelet count, at least under short-term exposure conditions. This is consistent with our hemostasis

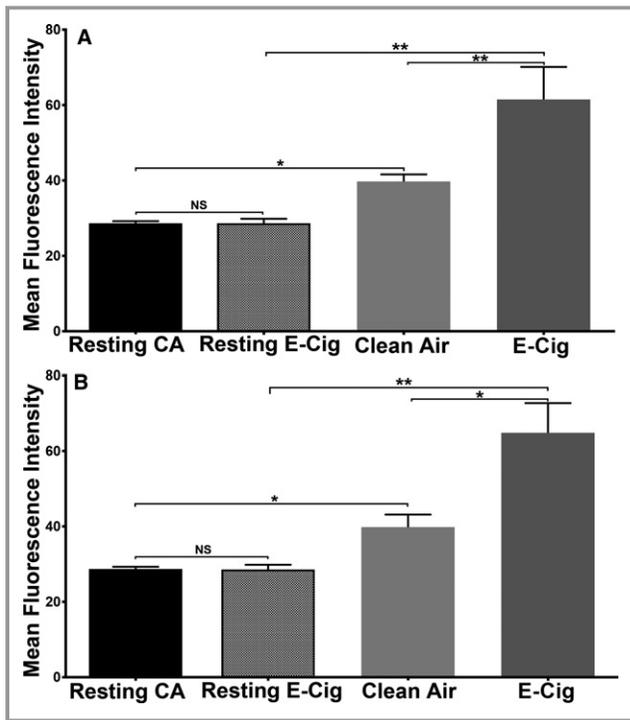


Figure 5. E-cigarette exposure enhances integrin activation. Platelets from e-cigarette- and clean air-exposed mice were washed, before stimulation with 10 $\mu\text{mol/L}$ ADP (A) or 5 $\mu\text{mol/L}$ U46619 (B). Platelets were incubated with fluorescein isothiocyanate-conjugated JON/A antibody, and the fluorescent intensities were measured by flow cytometry. Average mean fluorescence intensities shown (* $P < 0.05$; ** $P < 0.01$; NS, non-significant). Each experiment was repeated 3 times, with blood pooled from at least 5 to 6 mice each time. CA indicates clean air; E-Cig, E-Cigarettes.

findings, as a (significant) decrease in platelet count would be expected to result in a bleeding diathesis, and not the shortened bleeding time we observed. As for the effects of traditional tobacco, studies are controversial, with some showing the platelet counts to be increased⁵¹ or unaffected.^{52,53} These differences could be attributed, in part, to the different exposure conditions and study populations/subjects.

In light of the enhanced thrombosis risk and altered hemostasis in the e-cigarette-exposed mice, we next sought to investigate the mechanism by which these effects may manifest. Thus, we examined the e-cigarette platelet function/activation phenotype. Our results demonstrated that agonist-induced platelet aggregation, secretion (dense and α granules), and integrin and phosphatidylserine expression were enhanced as a result of e-cigarette exposure. These findings support the notion that e-cigarettes result in a state of platelet hyperactivity, which underlies, at least in part, the prothrombotic phenotype in the exposed mice. Additionally, these data are consistent with those seen in the e-cigarette vapor extract-exposed human platelet¹⁶ studies, in which

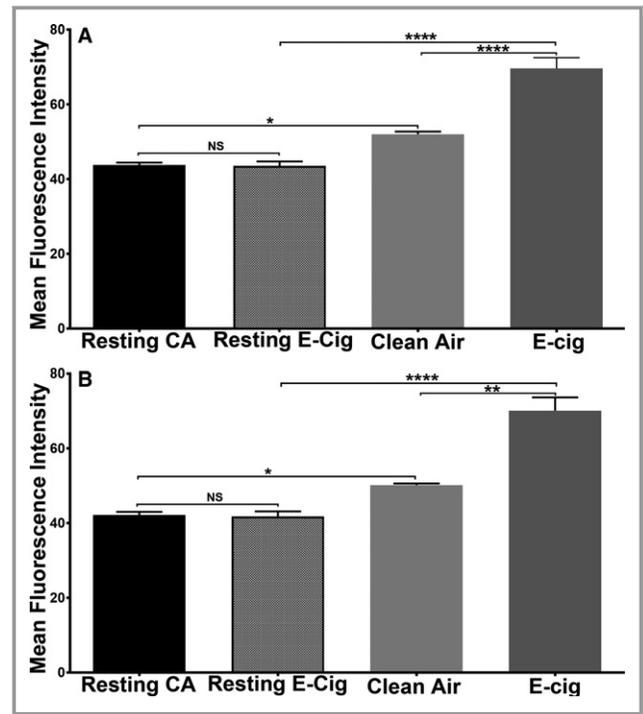


Figure 6. E-cigarette exposure enhances phosphatidylserine expression. Platelets from e-cigarette- and clean air-exposed mice were washed, before stimulation with 10 $\mu\text{mol/L}$ ADP (A) or 5 $\mu\text{mol/L}$ U46619 (B). Platelets were incubated with fluorescein isothiocyanate-conjugated Annexin V antibody, and the fluorescent intensities were measured by flow cytometry. Average mean fluorescence intensities shown (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; NS, non-significant). Each experiment was repeated 3 times, with blood pooled from at least 5 to 6 mice each time. CA indicates clean air; E-Cig, E-Cigarettes.

several functional responses were enhanced (eg, aggregation). Moreover, our data revealed that the e-cigarette-exposed platelets are less sensitive to inhibition by PGI_2 , in comparison to those from clean air; which may have also contributed to the thrombosis phenotype in these mice. Importantly, these results are also “similar” to those observed with traditional tobacco smoke, which heightens platelet activation (eg, aggregation)^{17,94} and renders platelets less sensitive to PGI_2 .⁹⁵ On the other hand, we observed no difference in the activation state of leukocytes between e-cigarettes and clean air. This finding suggests that leukocytes do not contribute to the observed e-cigarette phenotype; at least under the present experimental conditions. Given that traditional tobacco is known to enhance leukocyte activation,^{43,44} it is possible that longer e-cigarette exposures, and/or increasing the number of puffs or amount of nicotine concentration would have produced an effect. These issues will be the subject of future studies. To further delineate the mechanism behind e-cigarette-induced thrombogenesis and hyperactive platelets, we investigated the activation state/levels of biochemical markers of platelet function. Our

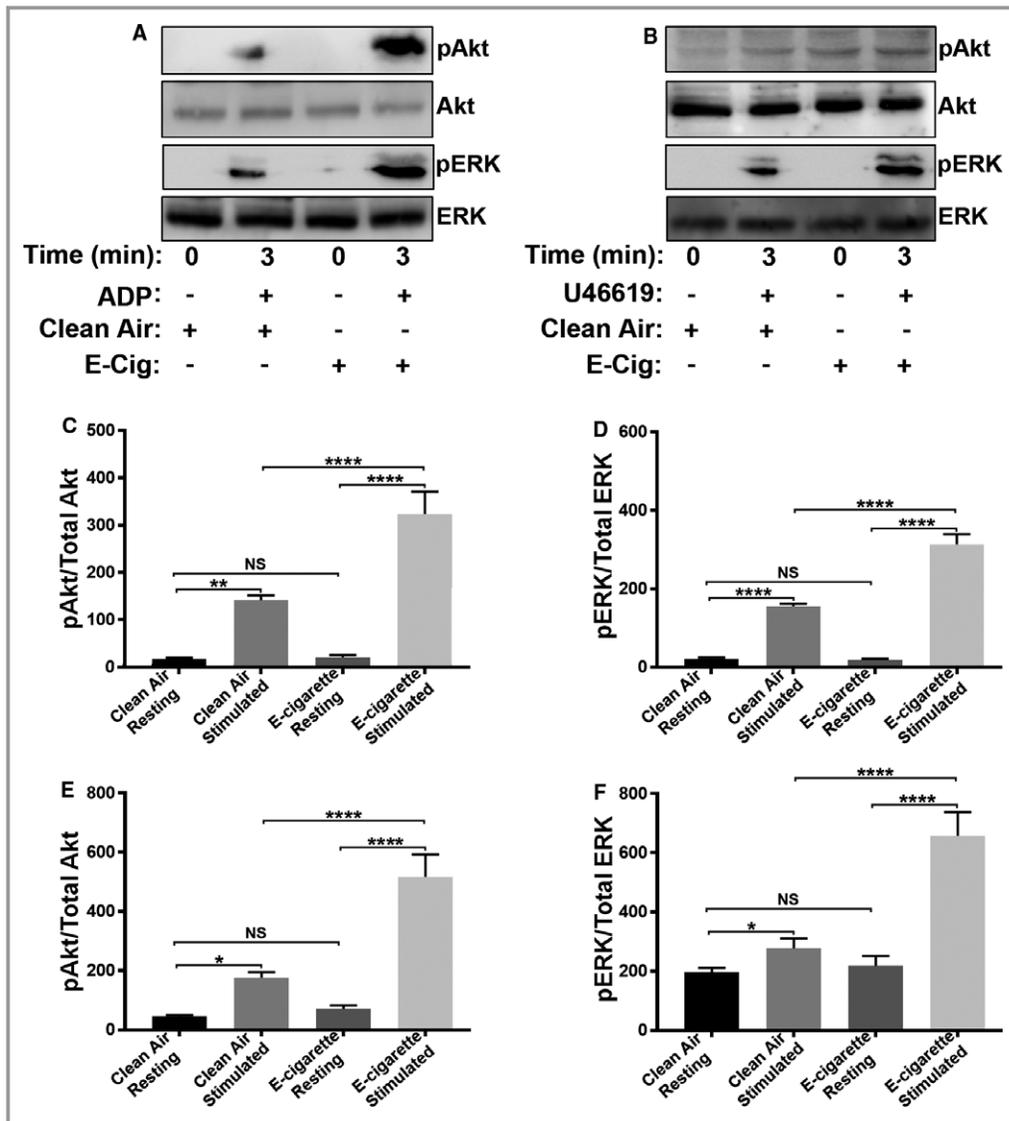


Figure 7. E-cigarette exposure enhances Akt and ERK activation (phosphorylation). Platelets from e-cigarette- and clean air-exposed mice were prepared, count adjusted to 1×10^9 , stimulated with 2.5 $\mu\text{mol/L}$ ADP (A) or 5 $\mu\text{mol/L}$ U46619 (B) for 3 minutes, and proteins were lysed using 1X sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being subjected to immunoblotting with anti-Akt, anti-pAkt, anti-ERK, and anti-pERK antibodies. Data were quantified using densitometric analysis with ImageJ, and presented as bar graphs for pAkt and total Akt (C and E), and for pERK and total ERK (D and F). Data are mean \pm standard error of mean of 3 independent experiments (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; NS, nonsignificant). E-Cig indicates E-Cigarette.

findings showed an increase in the levels of phosphorylated Akt and ERK in the e-cigarette-exposed mice. These findings suggest that the $G_{12/13}$ ⁹⁶ and G_i ⁹⁷ signaling pathways are involved, respectively, and provide biochemical evidence of the hyperactive state of platelets. These data are also consistent with previous studies in which both the Akt⁹⁸ and ERK^{99,100} pathways were upregulated by nicotine.

As for the constituents that e-cigarettes emit and that could be responsible for the observed platelet hyperactive state, they are presently unknown and under investigation. To this end, e-cigarettes are now known to be a source of a large

number of toxicants, such as nicotine, cotinine, aldehydes (eg, acrolein), and particulate matter.^{101–104} It is noteworthy that exposure to some of these toxicants, albeit from traditional tobacco smoking and other sources, was found to be associated with an enhanced platelet activation phenotype.⁶ Furthermore, while the effects of nicotine on platelet activation/aggregation are still controversial,^{16,105–108} particulate matter^{109,110} and acrolein⁹³ were indeed found to enhance platelet function.

In conclusion, our studies using a validated animal exposure model constitute the first investigation of the impact of short-

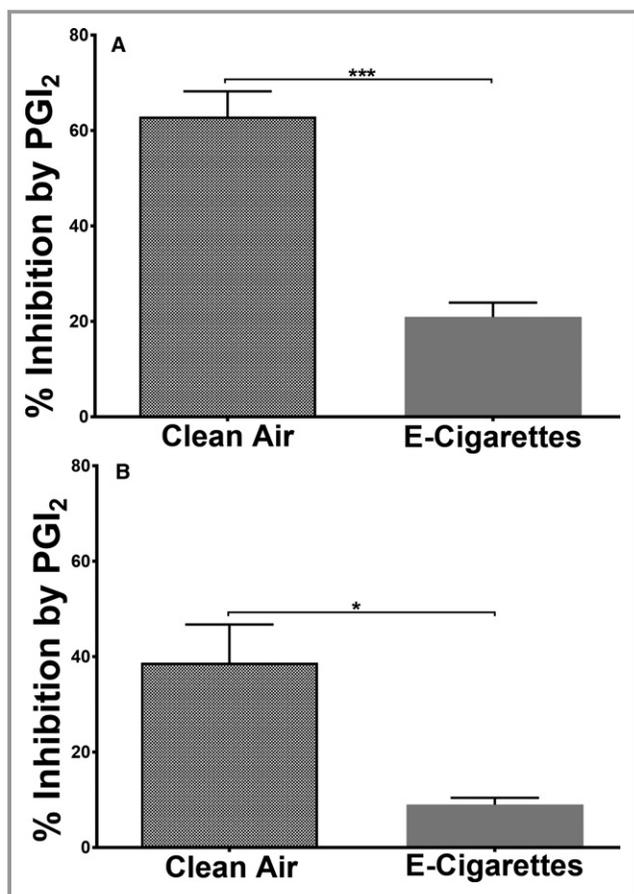


Figure 8. E-cigarette exposure renders platelets resistant to inhibition by prostacyclin (PGI₂). Platelets from e-cigarette- and clean air-exposed mice were incubated without or with PGI₂ (5 nmol/L) for 3 minutes before being stimulated with 2.5 μmol/L ADP (A) or 2 μmol/L U46619 (B), and their aggregation response was monitored using an aggregometer. The difference in aggregation between platelets exposed to clean air and e-cigarettes in the absence or presence of PGI₂ was calculated and plotted as a “percentage inhibition by PGI₂.” Each experiment was repeated 3 times, with blood pooled from at least 5 to 6 mice each time (**P*<0.05; ****P*<0.001).

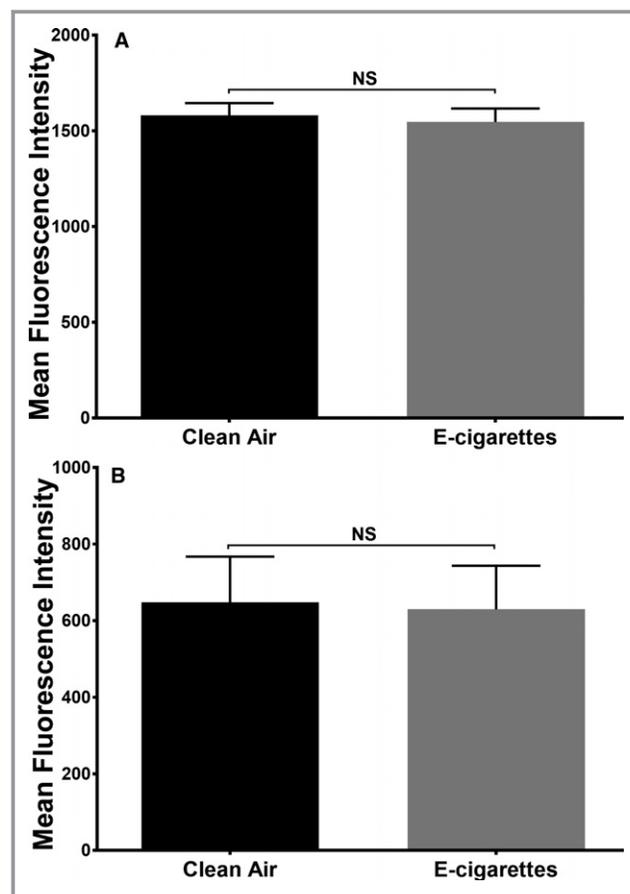


Figure 9. E-cigarette exposure does not affect leukocyte activation. Blood was collected from e-cigarette- and clean air-exposed mice and incubated with (A) phycoerythrin-conjugated anti-CD69 or (B) fluorescein isothiocyanate-conjugated anti-CD45. The fluorescent intensities were measured by flow cytometry. Average mean fluorescence intensities shown (NS, nonsignificant). Each experiment was repeated 3 times, with blood pooled from at least 5 to 6 mice each time.

whether e-cigarettes are a safer substitute for tobacco needs further investigation and will be the subject of future studies.

term whole-body e-cigarette exposure on platelet function and document for the first time that e-cigarettes do increase the risk of thrombosis. Moreover, our data support the notion that the elevated thrombotic risk is due, in part, to platelet hyperactivity, as well as decreased sensitivity to the physiological inhibitor PGI₂. Importantly, these findings are not only expected to increase awareness of the negative health consequences of the increasingly popular e-cigarettes but also undermine the assumption regarding their “high safety” profile. Our results should also shape policy development for evidence-based tobacco control (including e-cigarettes) and guide the Food and Drug Administration revisions to the legislation that would extend their regulations to e-cigarettes, as well as highlight the need for prevention of exposure to this form of tobacco. Finally,

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Disclosures

None.

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