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RESEARCH ARTICLE | *Electronic Cigarettes: Not All Good News?*

Acute exposure to e-cigarettes causes inflammation and pulmonary endothelial oxidative stress in nonsmoking, healthy young subjects

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Chatterjee S, Tao JQ, Johncola A, Guo W, Caporale A, Langham MC, Wehrli FW. Acute exposure to e-cigarettes causes inflammation and pulmonary endothelial oxidative stress in nonsmoking, healthy young subjects. *Am J Physiol Lung Cell Mol Physiol* 317: L155–L166, 2019. First published May 1, 2019; doi:10.1152/ajplung.00110.2019.—The effects of e-cigarette (e-cig) aerosol inhalation by nonsmokers have not been examined to date. The present study was designed to evaluate the acute response to aerosol inhalation of non-nicotinized e-cigarettes in terms of oxidative stress and indices of endothelial activation in human pulmonary microvascular endothelial cells (HPMVEC). Ten smoking-naïve healthy subjects (mean age ± SD = 28.7 ± 5.5 yr) were subjected to an e-cig challenge, following which their serum was monitored for markers of inflammation [C-reactive protein (CRP) and soluble intercellular adhesion molecule (sICAM)] and nitric oxide metabolites (NOx). The oxidative stress and inflammation burden of the circulating serum on the vascular network was also assessed by measuring reactive oxygen species (ROS) production and induction of ICAM-1 expression on HPMVEC. Our results show that serum indices of oxidative stress and inflammation increased significantly ($P < 0.05$ as compared with baseline), reaching a peak at approximately 1–2 h post-e-cig aerosol inhalation and returning to baseline levels at 6 h. The circulatory burden of the serum (ICAM-1 and ROS) increased significantly at 2 h and returned to baseline values 6 h post-e-cig challenge. ROS production by HPMVEC was found to occur via activation of the NADPH oxidase 2 (NOX2) pathways. These findings suggest that even in the absence of nicotine, acute e-cig aerosol inhalation leads to a transient increase in oxidative stress and inflammation. This can adversely affect the vascular endothelial network by promoting oxidative stress and immune cell adhesion. Thus e-cig inhalation has the potential to drive the onset of vascular pathologies.

e-cigarette; inflammation; oxidative stress; pulmonary endothelial activation; reactive oxygen species

INTRODUCTION

The use of electronic cigarettes (e-cig) has been growing rapidly among the US population, particularly among youth and young adults (49). This is an unsettling trend given the limited information on the effects of aerosolized components and the thermal degradation products contained in e-cig vapor. To date, there is little evidence to support the widespread perception that e-cigs are a safer alternative to tobacco cigarette smoking. Furthermore, recent evidence has demonstrated that regardless of nicotine levels, inhalation of e-cig aerosol alone has multiple adverse health impacts (48, 50, 61, 64).

Studies elsewhere on the exposure to fine (structures of <2.5 μm in diameter) and ultrafine particles (UFPs; structures of <0.1 μm diameter) originating from environmental pollution have shown detrimental outcomes on cardiovascular health (77). Once UFPs are inhaled, they are taken up by the alveoli, from where they translocate into the vascular space (see, for instance Ref. 51). Similarly, aerosolization of e-cig liquid generates UFPs (60) as well as a variety of chemical species that can potentially provoke a vascular endothelial activation response. Thus, e-cig aerosol inhalation may parallel the effect of environmental pollutants in activating inflammation, oxidative stress, and initiating the process of endothelial dysfunction (EDF). Hallmarks of EDF are increased generation of reactive oxygen species (ROS), decrease in nitric oxide (NO) generation, and formation of cellular adhesion molecules. EDF is generally considered one of the earliest events of atherogenesis, ultimately leading to cardiovascular disease (32 and references therein).

Thus far, the effects of non-nicotinized e-cig aerosol inhalation have not been studied in healthy individuals in terms of elevated blood markers of vascular inflammation and oxidative stress. This information is crucial in evaluating overall oxidative burden post e-cig aerosol inhalation. Of particular relevance is the investigation of the pulmonary vascular endothelial response to e-cig aerosol inhalation. In other words, would the oxidative stress and inflammation burden in the circulating blood "activate" the endothelial cells of the vascular network? To address these questions, we examined smoking-naïve subjects post-non-nicotinized e-cig aerosol inhalation by monitoring serum C-reactive protein (CRP), NO, NO metabolites (NOx), and intercellular adhesion molecules (ICAM-1). Elevated levels of serum CRP and decreased levels of nitric oxide metabolites have been associated with oxidative stress, while the increase in ICAM-1 is considered an index of inflammatory potential of the vasculature in the context of vascular disease (21). CRP, a protein made in the liver, is induced upon tissue inflammation and injury and is widely accepted as a marker of

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vascular inflammation, specifically in the context of atherosclerotic cardiovascular disease (37, 53). CRP levels are low in healthy individuals, and elevated blood CRP is indicative of acute inflammation and injury (8, 58). NO is well established to play an important role in vascular function and in maintenance of vascular tone (via vasodilation) (5, 45), and thus, NO levels are considered an index of vascular health. Indeed, serum NO metabolites (nitrite + nitrate = NOx) have been used routinely as a surrogate for NO production (23, 39, 79). ICAM-1, an endothelial protein induced upon inflammation, imparts an adhesive property to the endothelium, thus facilitating the adherence of leukocytes and other immune cells to the vascular endothelium. ICAM-1, when cleaved proteolytically, results in liberation of soluble ICAM-1 (sICAM-1; or sICAM) into the circulation. Serum sICAM levels reflect inflammation and immune system activation and are considered to be a diagnostic of various vascular inflammatory pathologies (19, 66). Importantly, circulating CRP, NO metabolites, and sICAM have been implicated in the progression of cardiovascular disease (9, 80).

Here, we addressed the hypothesis that the above markers of endothelial dysfunction are formed acutely following e-cig aerosol inhalation by monitoring the time course of the buildup of serum CRP, sICAM, and NOx levels post-e-cig aerosol inhalation in nonsmoking (smoking naïve) subjects. We report that there is a peak window of response post-e-cig inhalation, where oxidative stress in non-smoking subjects is comparable with that observed in regular e-cig smokers.

MATERIALS AND METHODS

E-cigarettes and laboratory reagents. E-puffer eco-disposable e-cigs were purchased from E-Puffer (New York, NY). E-puffer was chosen as it is a brand of non-nicotinized e-cig that is popular among young adults. Other brands that are popular lacked nicotine-free versions. The device consists of a cylindrical lithium battery that supplies 3.7 V to a dual-coil atomizer (heating coil) with a resistance of 2.7 ohms. The liquid tank attached to the atomizer is 1.3 ml in volume and is filled with e-liquid composed of 70% pharma-grade propylene glycol (PG) and 30% vegetable glycerine (VG). The atomizer temperature in a similar device has been reported to vary between 145 and 334°C (16).

Purified water from Millipore Nanopure Water systems was used for all assays. CellROX Green was from Life Technologies (Eugene, OR). Vacutainer tubes were from BD Biosciences, (Franklin Lakes, NJ). Quantikine ELISA kits for CRP and ICAM-1 were from R&D Systems (Minneapolis, MN). The colorimetric assay kit for nitric oxide metabolites (nitrate/nitrite) was purchased from Cayman Chemical (Ann Arbor, MI). Monoclonal anti-ICAM antibody was from Abcam (Cambridge, MA), and secondary antibody conjugated to Alexa 488 was Invitrogen (Life Technologies, Eugene, OR). Vectashield antifade mounting medium with DAPI was from Vector Laboratories (Burlingame, CA).

Study design. The study was approved by the Institutional Review Board of the University of Pennsylvania School of Medicine. All subjects enrolled were adults, and their written, informed consent was obtained prior to participation. Blood sample collection and experiments were carried out in accordance with relevant guidelines and regulations of the University of Pennsylvania School of Medicine. The study is registered at ClinicalTrials.gov (March 27, 2018; identifier: NCT03479203).

Subject recruitment for e-cigarette vaping challenge. Ten healthy nonsmokers of both sexes ranging in age from 23 to 33 yr of age (means ± SD of 28.7 ± 5.5) and BMI of ~18.5–30 were enrolled. Six regular e-cigarette smokers were also enrolled, but they were not subjected to the e-cig inhalation protocol. Subjects were selected from a pool of individuals who had participated in previous studies in the Department of Radiology and who had also agreed to be contacted for possible participation in future studies. Excluded were pregnant women, and individuals with cancer, HIV, mental illness, overt cardio- or neurovascular disease (prior myocardial infarction, stroke, transient ischemic attacks), serious arrhythmias, bronchospastic disease, and upper respiratory tract infection (past 6 wk). Also excluded were subjects with asthma and other similar respiratory diseases. Prior to the vaping challenge, subjects were asked to complete a questionnaire to ensure verification of the inclusion and exclusion criteria.

E-cig aerosol inhalation protocol. This protocol was designed based on reports of the average e-cig puffing topography in young adults. In general, the average "drag" or puff time on an e-cig device is between 2 and 5 s (70), but studies show that regular e-cig smokers puff or drag for longer duration than naïve smokers (27). Therefore, we reasoned that 2 s of puff time would better represent actual naïve human exposure. Subjects were instructed to drag and inhale in a standardized fashion in the presence of the research coordinator. The paradigm consisted of 16–17 inhalations or puffs, each 2 s long, during which subjects did not breathe in through the nose. The total time spent on the entire protocol (including inhalation, release of vapor, and a few seconds between puffs) was ~3 min and is equivalent to smoking an entire conventional cigarette. Overall, the protocol represents the average e-cig puffing topography in young adults (7).

Sample (blood) collection and storage (serum). Two milliliters of blood were drawn before and at various intervals post-vaping (Fig. 1) and serum isolated. Briefly, blood was collected in vacutainer tubes and serum obtained after centrifugation (1,000–2,000 *g* for 10–15 min) post-clot formation. Serum was then stored in eight to 10 aliquots at −80°C before analysis. Each aliquot was used for a single assay, and samples were never subjected to freeze-thaw cycles.

Assessment of biomarkers CRP and ICAM in serum. Aliquots of serum were thawed immediately before analysis. CRP and ICAM-1 were detected using commercially available kits (Quantikine R&D Systems, Minneapolis, MN). Briefly, samples or standards were added to microplates precoated with CRP or ICAM-1 antibody, respectively. Antibodies would then bind to CRP or ICAM-1 in the samples and standards. The unbound substances were washed and removed, and a second antibody specific to CRP or ICAM-1 was added. The amount of the second antibody that binds in the microplates is proportional to the CRP or ICAM-1 amounts present in the initial samples. This

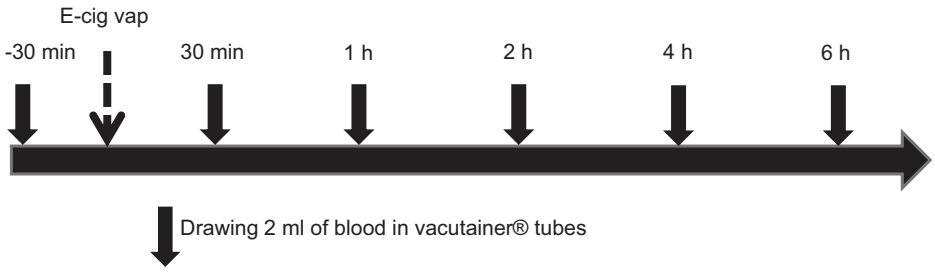


Fig. 1. Schema of blood collection from smoking-naïve subjects at various time points pre- and post-non-nicotinized e-cig inhalation.

antibody is conjugated to horseradish peroxidase; thus, a substrate solution that detects horseradish peroxidase activity and that changes color upon binding was used to monitor CRP and ICAM, respectively. Both CRP and ICAM-1 analyses were completed in triplicate and data averaged for all 10 subjects. All data and background microplate values read out by the ELISA plate reader were checked by two separate laboratory personnel.

Assessment of nitric oxide metabolites (nitrate + nitrite, NOx) in serum. The measurement of nitrate/nitrite concentration in serum is used as an index of NO production in the body (39, 79). The concentration of NOx is assessed by using the Griess reaction (34). Here, NOx in serum was measured using the commercially available Nitrate/Nitrite Colorimetric Assay kit. Briefly, 40 μl aliquots of serum were diluted, and the nitrate in this aliquot was converted to nitrite. Next, Griess reagent was added to obtain an azo compound that was measured by absorbance colorimetry. Standards provided by the manufacturer were used to quantify NOx as serum.

Cell Culture. Immortalized human pulmonary microvascular endothelial cells (HPMVEC; clone ST1.6R) were gifted to one of the authors (S. Chatterjee) by Prof. C. J. Kirkpatrick, as described previously (41). HPMVEC were plated in Greiner Bio-One Cell View Dishes (with glass bottom to enable fluorescence imaging) and cultured using Medium-199 supplemented with 15% fetal bovine serum (FBS), glutamax, antibiotics, and endothelial cell growth supplement (Millipore Upstate). HPMVEC were monitored for ICAM-1 induction and (in separate experiments) for ROS production. Both ICAM-1 and ROS were monitored by incubating HPMVEC using medium containing 15% serum (as collected in Schema 1) from subjects pre- and post-e-cig challenge. Two hours postincubation, the medium was replaced with fresh medium (with FBS). To measure ICAM-1, HPMVEC were fixed in 4% paraformaldehyde, followed by permeabilization. Permeabilized HPMVEC were immunostained using anti-ICAM (1:250) for 2 h. The secondary antibody used was anti-mouse IgG conjugated to Alexa 488 (green). Cells on glass coverslips were mounted on slides using vectashield antifade mounting medium with DAPI (for nuclear counterstaining). Imaging was done using a Leica STED super-resolution laser microscope with excitation and emission filters for λ_{ex} at 488 nm and λ_{em} at 500–560 nm (maximum ~530 nm). All images were acquired using the same setting and LAS X software. For monitoring ROS, serum-treated HPMVEC were labeled with a ROS-sensitive dye (CellROX Green, 5 μM for 30 min) and imaged by epifluorescence microscopy using a Nikon TMD epifluorescence microscope equipped with a Hamamatsu ORCA-100 digital camera and Metamorph imaging software (Universal Imaging, West Chester, PA). Images were acquired at λ_{ex} = 488 nm using both ×4 and ×20 lenses. All images were acquired with the same exposure and acquisition settings as reported previously (10, 73).

Monitoring ICAM-1 expression in HPMVEC using fluorescence microscopy. HPMVEC were treated with serum from subjects exposed to e-cig aerosol. These cells had been established in prior work to represent human lung endothelium (38). The endothelial purity of each batch was confirmed by labeling with endothelial dyes, as described earlier (15). Postincubation with medium supplemented with 15% serum (as collected in Schema 1), HPMVEC were monitored for ICAM-1. Two hours postincubation, HPMVEC were immunostained for ICAM-1 and imaged by fluorescence microscopy. Appropriate controls were nonimmune IgG and no-antibody treated samples. Expression of ICAM-1 was quantified from fluorescent micrographs by integrating the fluorescence intensity using LasX and ImageJ software.

Monitoring ROS production by HPMVEC using fluorescence microscopy. ROS production by HPMVEC was monitored using a ROS-sensitive dye (CellROX Green) and imaged by fluorescence microscopy, as reported in some of the authors' prior studies (59, 76). CellROX Green is nonfluorescent under control conditions but reacts with ROS (and free radicals) to get oxidized to a green fluorophore. Green fluorescent signal in cells post-CellROX treatment is well accepted as indicative of ROS/oxidative stress (1, 24). To ascertain the source of cellular ROS, separate experiments were carried out using NADPH oxidase 2 inhibitor apocynin. HPMVEC were preincubated with 300 μM apocynin for 1 h before incubation with serum.

Quantification of data, sample size, and statistical analysis. Quantification of fluorescent intensity of images was carried out using LasX (for Leica STED images), MetaMorph for Nikon microscope images (Molecular Devices, Sunnyvale, CA), or ImageJ (National Institutes of Health, Bethesda, MD) analysis software. The integrated intensity was measured within the entire field and background fluorescence subtracted using the threshold tool, as reported earlier (10, 54). Data were expressed either in arbitrary fluorescence units or in multiple (*n*-fold) increases over untreated or pre-cig challenge controls. Data were expressed as means ± SD, and one-tailed, paired *t*-tests were used to determine statistical significance.

To detect a significant difference in NOx between pre- and post-e-cig exposure (the latter pertaining to multiple time points), a standardized effect size of 1.0 would be required in a paired *t*-test for 80% power at $\alpha = 5\%$ in 10 subjects. Effects of this order have been observed in similar experiments.

RESULTS

E-cig aerosol inhalation led to a transient increase in blood markers of inflammation and injury. Serum CRP levels were found to increase between 60 and 120 min but decreased thereafter. Figure 2*A* shows the CRP levels plotted for each subject over time from −30 to 360 min post-e-cig challenge. Of note is that baseline values differed between subjects

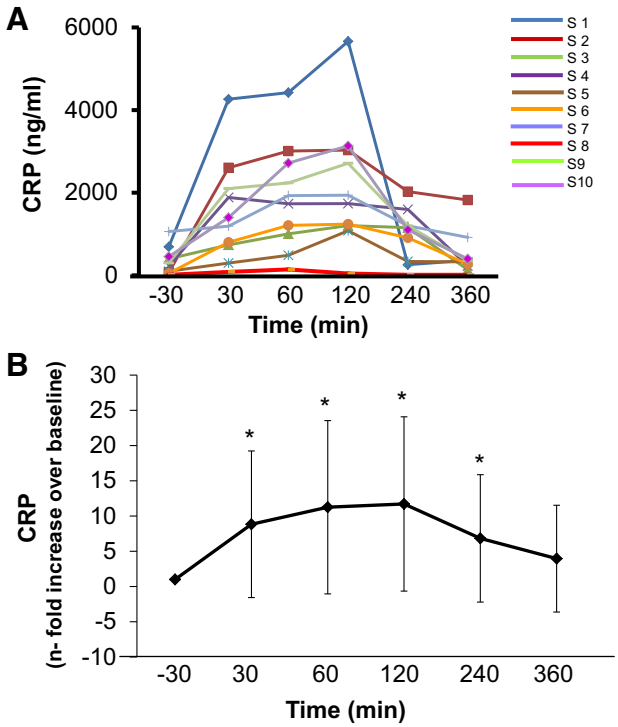


Fig. 2. C-reactive protein (CRP) in serum with e-cigarette (e-cig) inhalation. *A*: profile of CRP in the serum of all subjects post e-cig vaping. Blood was collected before (−30 min) and postvaping (30–360 min) from 10 subjects (*S1–S10*). CRP was assayed using quantitative ELISA and quantified using reference curves of standards provided by the manufacturer (R&D Biosystems). *B*: quantification of CRP levels across all subjects postvaping. For each time point, the CRP levels for all 10 subjects were expressed as *n*-fold increase over the respective baseline (i.e., the respective prevaping levels). Data are expressed as means ± SD. *$P < 0.05$ as compared with baseline.

(among the 10 subjects, the baseline CRP values ranged from 14 to 690 ng/ml). Furthermore, there was a wide variation in the magnitude in CRP response (maximum levels varied between ~1,200 and 5,700 ng/ml) across subjects. The elevated serum CRP levels typically persisted until ~240 min post-e-cig challenge, declining rapidly thereafter. However the values did not revert to baseline at the final measurement time of 360 min. There was a large intersubject variation in the CRP increase profile post-e-cig challenge. Despite this variation, the overall increase in CRP expressed as fold increase over baseline was significant at 30, 60, 120, and 240 min post-e-cig challenge (Fig. 2*B*).

The baseline serum NOx values differed widely within the subjects (between 6 and 102 μmol/l or nmol/ml). These values showed a decline after 30 min post-e-cig aerosol inhalation (Fig. 3*A*) that continued until 120 min, beyond which the NOx concentrations were gradually restored to preinhalation values. Indeed, by 6 h post-e-cig aerosol inhalation, the NOx values were restored to within 80% of the respective baseline values. The aggregate serum NOx across all subjects (achieved by expressing NOx as *n*-fold change over control or pre-cig challenge values) displayed in Fig. 3*B* further supports the evidence that nitric oxide metabolites decreased significantly at the 60- ($P < 0.005$) and 120-min ($P < 0.05$) observation window following the e-cig challenge, returning to baseline values thereafter. In comparison with smoking-naïve subjects, regular e-smoking individuals showed an average (baseline) serum NOx value (across 6 subjects) of 16.6 ± 6.3 μmol/l.

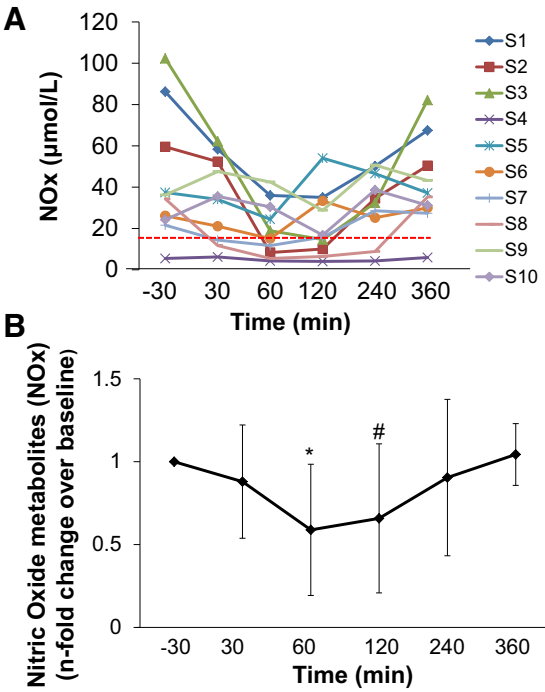


Fig. 3. Stable end products of nitric oxide (NO) oxidation, i.e., nitrate-nitrite [nitric oxide metabolites (NOx)] in serum with e-cig inhalation. *A*: profile of serum NOx at various time points pre- and post-e-cigarette (e-cig) inhalation for *subjects 1–10* (*S1–S10*). Dotted red line represents the average NO values for 6 regular e-cig smokers. Samples were assayed for NOx colorimetrically using a nitrite-nitrate Griess reaction assay, and NOx was quantified by standard curves. *B*: average fractional change in NOx pre- and post e-cig inhalation in all 10 subjects. For each subject, the NOx values were expressed as *n*-fold change over their respective baseline (i.e., the prevaping levels normalized to 1). *$P < 0.005$; #$P < 0.05$ as compared with baseline.

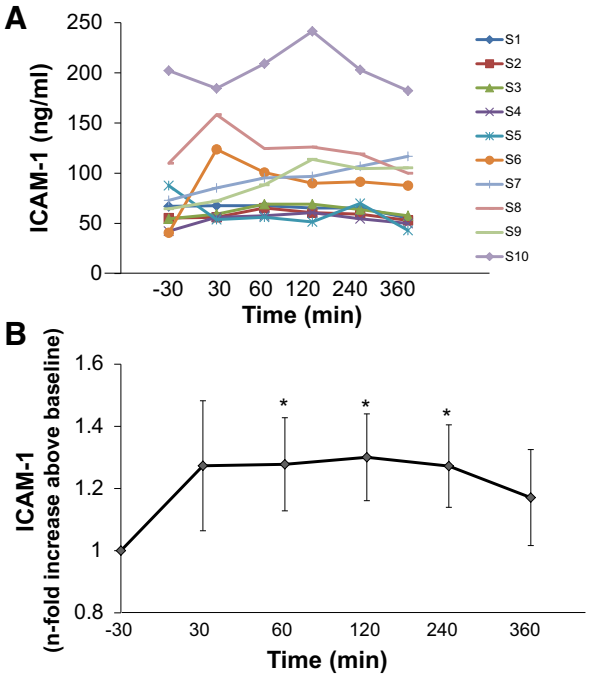


Fig. 4. Soluble intercellular adhesion molecule (sICAM) in serum with e-cigarette (e-cig) inhalation. *A*: time course of ICAM-1 in the serum of all subjects post-e-cig vaping, as assayed using quantitative ELISA. ICAM-1 was quantified using reference curves of standards provided by the manufacturer (R&D Biosystems). *B*: average ICAM levels in blood post-e-cig inhalation across all 10 subjects (S1–S10). Data in the form of means ± SD of ICAM refer to samples obtained from 10 subjects for different time points. ICAM-1 levels are expressed as *n*-fold increase above baseline (i.e., pre-inhalation levels normalized to 1). *$P < 0.05$ relative to baseline.

Soluble ICAM-1 (sICAM) shed into the circulation post e-cig aerosol inhalation is shown in Fig. 4*A*. Except for two outliers (S6 and S8), the increase seemed to be gradual between 30 and 240 min post-e-cig challenge. Across all subjects, sICAM buildup increase during the time course showed a modest (~1.3- to 1.4-fold over baseline) but significant increase between 60 and 240 min. The values reverted to (pre-inhalation) baseline at 6 h (Fig. 4*B*).

The cumulative effect of oxidative stress and inflammation potential of the serum on the vascular network was evaluated in terms of its effect on the endothelium. Inflammation and oxidative stress were evaluated by measuring ICAM-1 expression (Fig. 5) and ROS generation (Fig. 6) in HPMVEC treated with media supplemented with serum pre- and post-e-cig challenge. ICAM-1 expression in HPMVEC treated with serum pre-inhalation was very low (at near undetectable levels in fluorescence micrographs; Fig. 5*A*) and showed an increase only after treatment with serum obtained 120 min post-inhalation (Fig. 5, *A* and *B*). Images acquired at higher magnification show a perinuclear green stain, indicating an increase in induction of cellular ICAM-1 (Fig. 5*A*, *inset*). Although there was some variation in ICAM-1 expression between subjects (specifically, *subjects S7* and *S9* showed a higher increase) post-e-cig serum treatment, cells treated with serum 6 h post-inhalation indicate a return to baseline values (Fig. 5*B*). Overall, ICAM-1 in cells showed a significant increase ($P < 0.001$

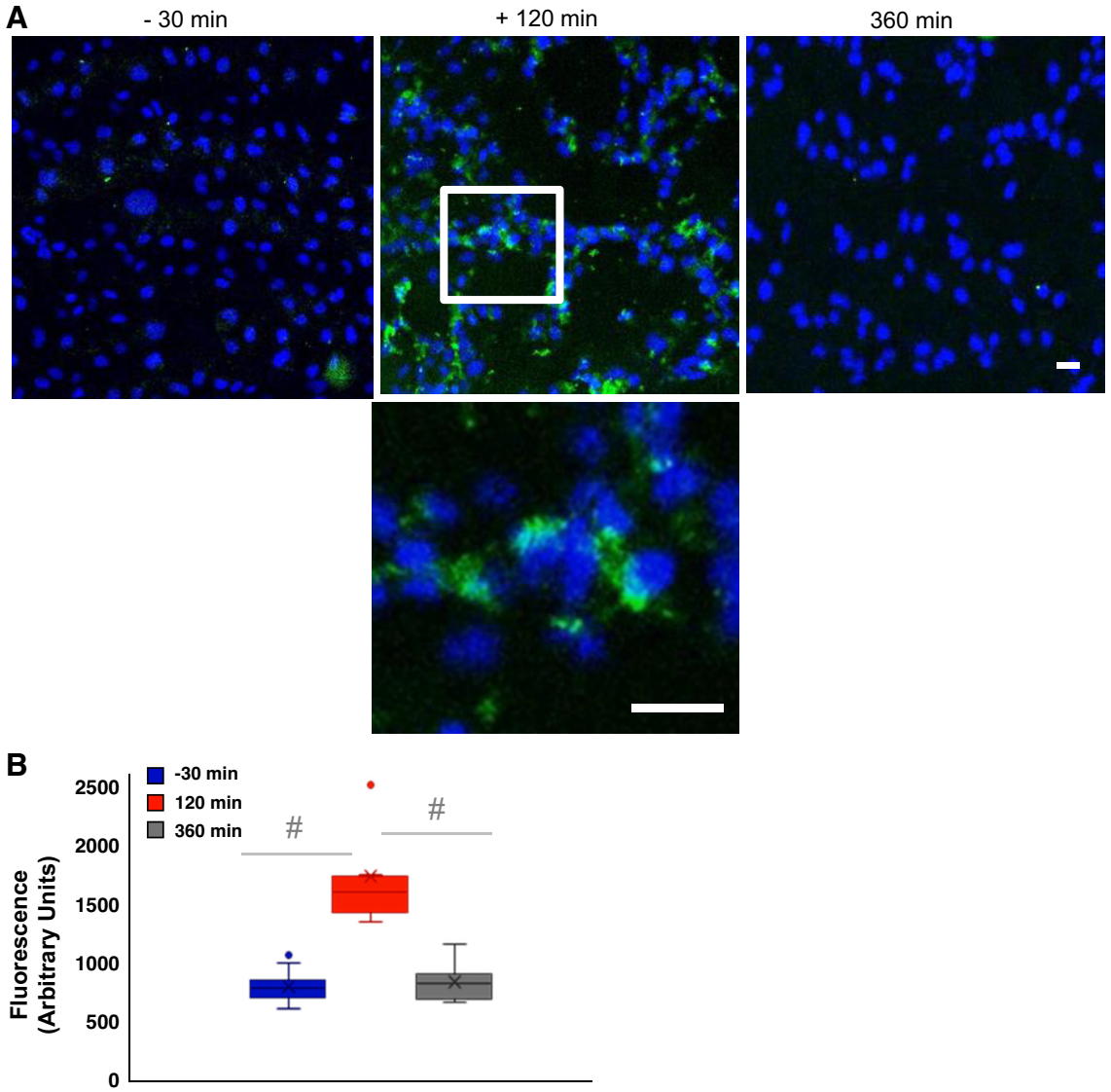
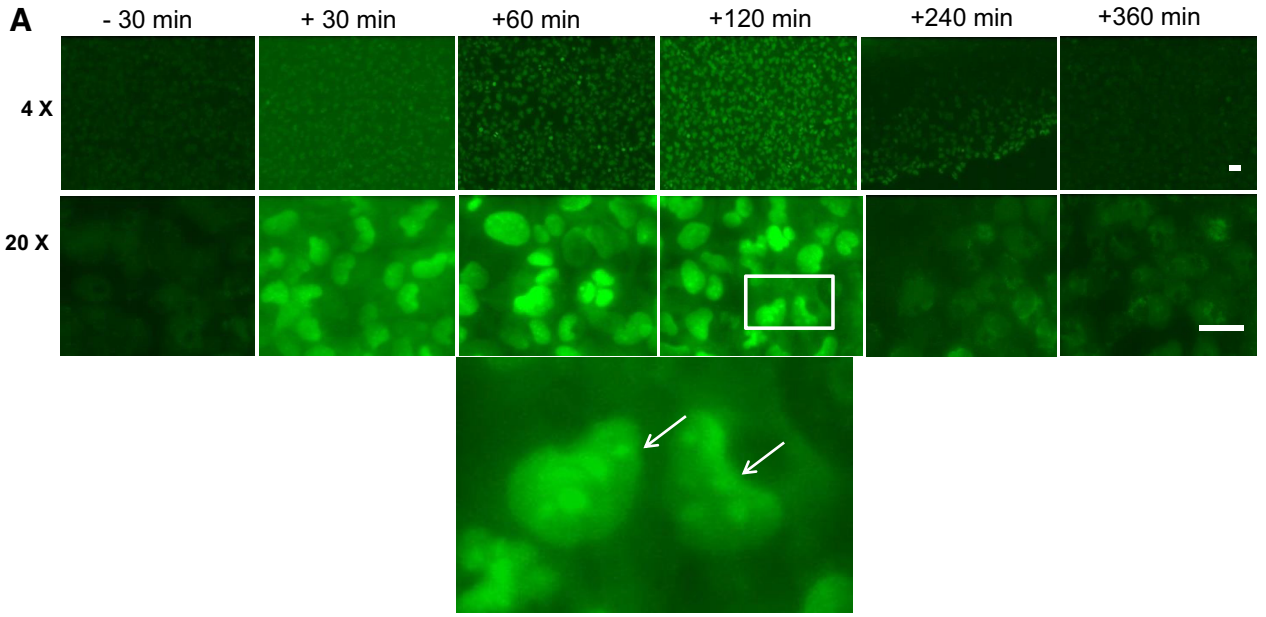


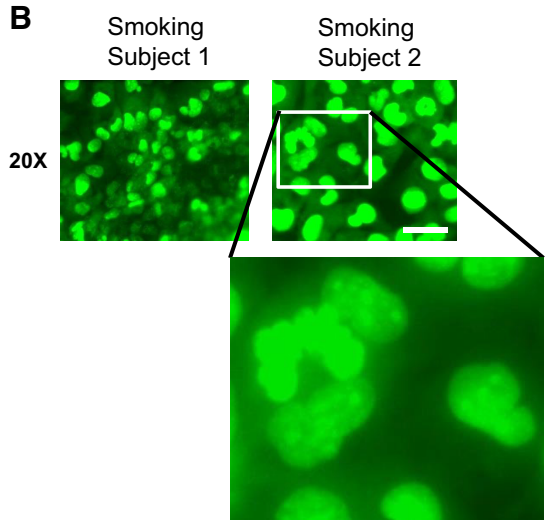
Fig. 5. Effect of serum on endothelial inflammation as monitored by intercellular adhesion molecule 1 (ICAM-1) expression on endothelial cells exposed to serum post e-cigarette (e-cig) inhalation. *A*: human pulmonary microvascular endothelial cells (HPMVEC) were exposed to media with 15% serum from subjects pre- and post-e-cig inhalation (120 and 360 min) and immunostained for ICAM (green). Blue, nuclear stained DAPI. Cells were imaged on Leica STED super resolution microscope at λ_{ex} laser line 488 nm, λ_{em} 500–560 (maximum ~530 nm). *Inset* (+120 min) is magnified below showing perinuclear staining. Scale bar, 25 μm. *B*: ICAM-1 expression on HPMVEC as obtained from quantification of fluorescence intensity of green signal, as observed in Fig. 4*A*. Images were quantified by integrating the fluorescence intensity and normalized to equal number of cells, as measured by the no. of nuclei (blue DAPI stain). Data are presented as a box and whisker plot of ICAM expression on HPMVEC upon serum treatment pre- (−30 min) and post-e-cig inhalation (120, 360 min) for 10 subjects. Within each box plot, the central horizontal line represents the median, whereas *top* and *bottom* lines represent the 1st and 3rd quartiles, respectively. X indicates the mean. Vertical lines (whiskers) represent the range of values. Outliers are beyond the end of each whisker and are displayed as dots. #$P < 0.001$, 120-min value as compared with values at −30 and +360 min.

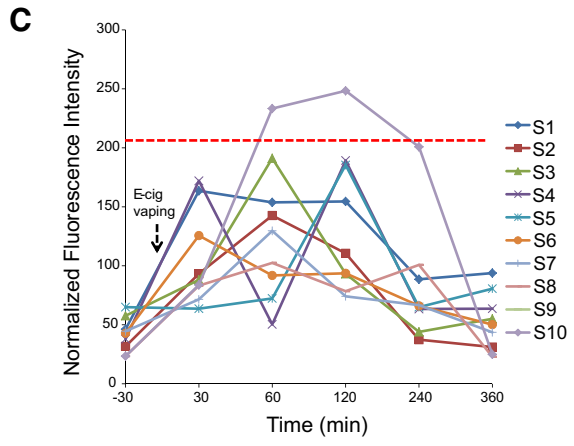
as compared with baseline values) upon treatment with serum obtained from subjects 120 min post-e-cig aerosol inhalation. Low to no background signal was obtained in the IgG and "no antibody" controls (data not shown).

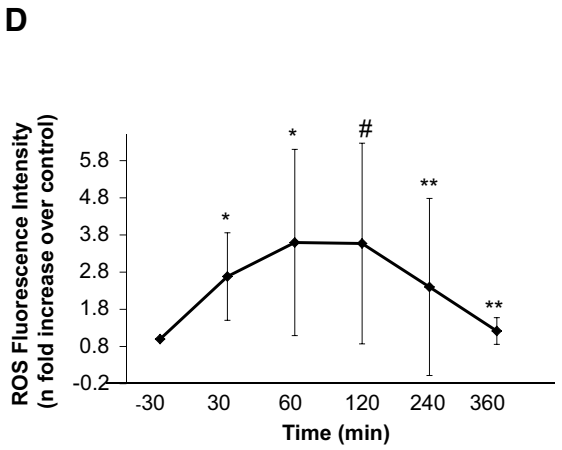
The amount of ROS generated by HPMVEC (as evidenced by the fluorescent signal) in response to serum varied substantially among subjects. Each subject showed a unique fluorescence signal profile. Images shown in Fig. 6*A* are representative of the effect observed in two subjects and indicate observable ROS production in HPMVEC exposed to serum 30–120 min post-e-cig challenge. The CellROX green dye used for ROS detection is a cell-permeable, nonfluorescent compound in its reduced form but becomes oxidized by ROS to emit green fluorescence upon excitation at 488 nm. Fluorescence micrographs in Fig. 6*A* show substantial nuclear signal due to the ability of the oxidized CellROX to bind the DNA. HPMVEC exposed to serum from regular e-cig smokers (Fig. 6*B*) produced ROS comparable with smoking-naïve subjects exposed to e-cig inhalation.

The time course of the fluorescent intensity of the green signal in cells exposed to serum is shown in Fig. 6*C*. Baseline ROS production in regular e-cig smokers (dotted line) is comparable with that generated by e-cig inhalation by smoking-naïve subjects. The aggregate ROS production across all









subjects displayed in Fig. 6*D* further supports that the endothelial ROS production was maximum during the chosen 30- to 240-min observation window following the e-cig challenge in smoking-naïve subjects.

Additional insight into the source of endothelial ROS production was obtained by pretreating HPMVECs with the NADPH oxidase 2 inhibitor apocynin. Earlier work from some of the present investigators' research had shown that NADPH oxidase 2 is the major source of ROS production in these and other endothelial cells of the pulmonary vasculature; they also reported that pretreatment with the NADPH oxidase inhibitor apocynin reduced ROS and downstream signaling in cells in vitro and in the vasculature in vivo (10, 13). Indeed, apocynin pretreatment of cells 60 min before serum exposure reduced ROS production at 60 min by ~75%, thus pointing to a role for NADPH oxidase activation as a major source of ROS generation by HPMVEC in response to serum treatment (see Fig. 7).

DISCUSSION

E-cigarette vaping is rapidly emerging as a potentially attractive alternative to traditional cigarette use. This is spurred by the notion of e-cig inhalation being safer than conventional nicotine delivery methods based on combustion of tobacco (25, 26, 57, 69). However, studies comparing the chemistry of aerosol generated by e-cigs with that of cigarette smoke have provided substantial evidence that vaporization (aerosolization) of e-cig fluid components (propylene glycol, vegetable glycerin, flavorings with or without liquid nicotine) and their thermal degradation products impart a toxicological burden that poses a potential health risk (17, 20, 35, 47). Studies to date on e-cig health effects have been somewhat inconsistent (28, 30, 61), which is due largely to differences in study design in terms of the e-cig products, variability in subject inclusion-exclusion criteria, e-cig vaping topography, and the biological parameters assessed. While products used in prior studies have varied from nicotinized and non-nicotinized as well as several flavored versions, biological assessments have in general measured cell damage in vitro in culture systems exposed to e-liquid (3, 50, 67, 68). Such models, although useful in understanding the effects of e-cig components on cells, like endothelial or airway epithelial cells, do not represent the dynamic environment of actual inhalation by a human smoker, which involves extensive interaction between the aerosol inhaled and the lungs. In vivo studies have generally been based on mouse models (33, 43). Of late, there have been several reports on the detrimental impact of e-cigs on respiratory and vascular responses in adults with daily e-cig use (29, 31, 36, 40). Although these studies elucidated both acute and long-term changes, the assessments were on intact vascular function (blood pressure and arterial stiffness in blood vessels (31). Furthermore, in some studies, subjects involved had a smoking history, thus confounding the effects of e-cig aerosol inhalation (40). Therefore, there is a lack of information on early inflammation and oxidative stress that accompanies acute inhalation of e-cig aerosol in nonsmoking individuals.

Here, we designed a study to evaluate oxidative stress and inflammation in the serum of nonsmoking (smoking-naïve) subjects in response to acute e-cig aerosol inhalation. Non-nicotinized e-cigs were chosen, as nicotine (the stimulant that drives gratification in smokers) delivery in e-cig is highly variable and the effect of nicotine itself would be a major confounder. The effects of non-nicotinized e-cigs need particular attention given that the physiological effects of nicotine are already fairly well understood, whereas the implications of the aerosol alone are not. In general, the e-cig puffing topography varies considerably among inhalers and ranges in puff clustered in groups of two to 20 (70); therefore, we used an average puffing maneuver comprising of 16–17 puffs (7). Following post-e-cig exposure, inhaled particles translocate from the alveoli into the vascular space, resulting in an elevation in inflammatory mediators in the blood. We chose to evaluate the response of the vascular system to such an insult by treating human pulmonary endothelial cells with media supplemented with human serum from e-cig smokers (in lieu of fetal bovine serum as used in some studies (4, 18, 82). Such studies based on applying serum (of diseased human subjects) to endothelial cells in culture and measuring the endothelial responses have been reported for other pathologies such as obstructive sleep apnea and Alzheimer's disease (63, 82).

Circulating CRP, an acute-phase plasma protein, is synthesized in response to general inflammatory episodes and is reported to be elevated in several pathologies, specifically vascular related changes (6, 21). In smokers, too, CRP levels parallel smoking status, and a dose-dependent correlation between CRP and smoking frequency has been reported (2, 74). In our study, CRP levels showed a steady increase between 30 and 120 min post-e-cig inhalation (Fig. 2). The large intersubject variations in absolute CRP levels postvaping possibly reflect differences in individual response to an oxidative challenge. Indeed, responses to oxidants have been found to depend upon an individual's antioxidant capacity (42). Studies show that e-liquid exposure increases expression of several antioxidant genes (such as *GCLC* and *GCLM*, which catalyze the production of the cellular endogenous antioxidant glutathione,

Fig. 6. Endothelial activation potential as monitored by reactive oxygen species (ROS) production by human pulmonary microvascular endothelial cells (HPMVEC) upon treatment with subjects' serum post e-cigarette (e-cig) vaping. *A*: ROS production in HPMVEC exposed to media with 15% serum from subjects post-e-cig vaping. Cells labeled with ROS-sensitive dye CellROX Green were imaged at low and high magnification on the stage of a microscope at λ_{ex} 488 nm, λ_{em} 500–560 (maximum ~530 nm). Images here are representative of ROS production by serum of 2 subjects (*subjects 1* and *3*). *Inset* is magnified below. Arrows show subcellular organelles. Scale bar, 10 μm. *B*: endothelial activation potential in serum from e-cig smokers. ROS was monitored in HPMVEC upon treatment with serum obtained from 6 regular e-cig smokers. Images here are representative of ROS production by serum of 2 subjects. Scale bar, 10 μm. *Inset* is magnified below. All images in *A* and *B* were acquired at the same settings using Metamorph Imaging Software. *C*: ROS production profile in individual subjects (*S1–S10*). Images acquired at ×20 magnification were used for quantification. Data represent means of three separate experiments. SD values are not displayed for ease of visualization. ImageJ software was used to integrate the fluorescence across an entire field. Background fluorescence was subtracted and data normalized to equal pixel area, as reported earlier (13, 54, 73). Red dotted line represents the average "baseline" values (210 ± 11.7) across a group of 6 regular e- cig smokers. *D*: quantification of ROS production by HPMVEC across all subjects post-e-cig vaping. For each subject, the fluorescence intensity (denoting ROS production) is expressed as *n*-fold increase over pre-e-cig vaping levels (baseline). Data across all 10 subjects were obtained and are expressed as means ± SD. $*P < 0.001$; $\#P < 0.01$; $**P < 0.05$ relative to baseline.

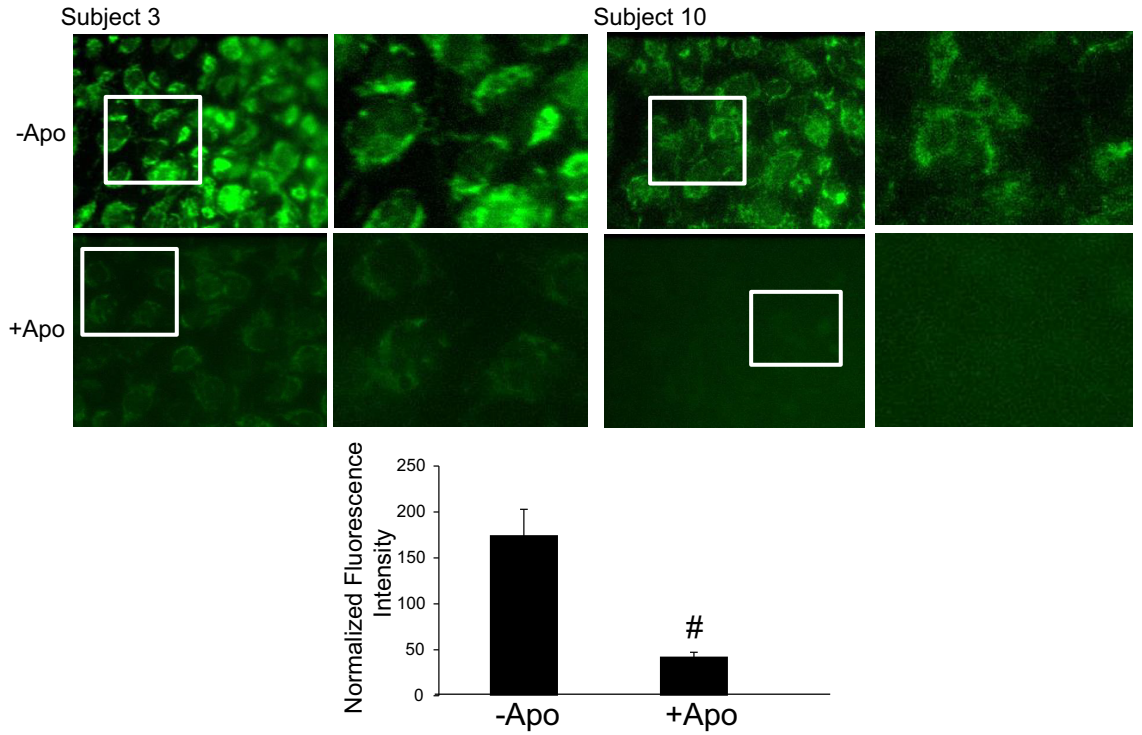


Fig. 7. Apocynin pretreatment reduces reactive oxygen species (ROS) production in response to exposure to serum from subjects post e-cigarette (e-cig) challenge. *Top*: human pulmonary microvascular endothelial cells (HPMVEC) were pretreated for 1 h with 300 μM apocynin in regular media before incubation with serum (from subjects 60 min post-e-cig challenge). Cells with (+Apo) and without apocynin (−Apo) were imaged for ROS under identical assay conditions. *Inset* is magnified to show ROS within the cellular regions. *Bottom*: fluorescence intensity (denoting ROS production) integrated across the entire microscopic field for all 10 subjects (in arbitrary fluorescence units) is expressed as means ± SD. #$P < 0.001$.

and glutathione peroxidase *GPX*, which scavenges H_2O_2 and other organic hydroperoxides) (68). The large degree of variation in pre-e-cig challenge values (70–1,000 ng/ml CRP) indicates differences in baseline antioxidant capacity. This, coupled with possible variation in antioxidant increase across subjects post-e-cig exposure, could account for the large intersubject variation observed.

Loss of NO is considered a key event for endothelial dysfunction (5, 45). However, NO measurement in the circulating blood poses a problem due to the radical's short half-life; therefore, its stable end products, nitrite and nitrate, are often used to evaluate NO production (39, 79). Because a strong correlation between NO production by the vasculature and serum NOx levels was reported earlier, measurement of NO metabolites are considered an index for the severity of endothelial dysfunction (46). NO is produced by the enzyme nitric oxide synthase (NOS) that oxidizes L-arginine to L-citrulline. Once produced, NO reacts with ROS (superoxide) to produce peroxynitrite ($ONOO^-$). Peroxynitrite is well established as an oxidizing and nitrating agent that reacts with a wide array of biomolecules such as DNA, proteins, and lipids (56). Because these reactions trigger cellular responses that can drive apoptosis and necrosis, $ONOO^-$ production is considered to be detrimental to cellular function (62). Because the production of peroxynitrite is accompanied by reduction in NO, it is also linked to endothelial dysfunction (12).

We observed that serum NOx levels varied across the 10 subjects (20–100 μM; Fig. 3). In general, NOx values have been found in the range of 40–60 μM and values <10 μM have been reported to correlate with vascular disease (44). The transient decline in NOx levels observed post-e-cig challenge (Fig. 3*A*) presumably arises from its reaction with ROS, which are concomitantly increasingly produced during the same period. Elsewhere, NO production has been reported to decline via reduced endothelial nitric oxide synthase (eNOS) expression or posttranslational regulation of eNOS activity; however, these have been chronic models of disease, unlike acute e-cig aerosol inhalation examined in this study (75). However in regular e-smokers, NOx values were low (~16 ± 6 μM, dotted line) and representative of an onset of vascular disease (<10 μM).

The inflammatory mediator monitored was ICAM-1; ICAM, when expressed on the endothelium, participates in localization and adherence of leukocytes. ICAM-1 on the endothelial cell surface can be proteolytically cleaved in response to endothelial activation and/or damage and the resultant soluble ICAM (sICAM) "shed" into the circulation (78). sICAM is well established as a candidate marker of vascular inflammation in disorders ranging from atherosclerosis and myocardial infarction to cancer (52, 72). In the present work, basal levels of ICAM-1 were found to vary across subjects presumably on account of their general health; however, all subjects showed an increase in the 30- to 240-min window and an eventual decline to pre-cig challenge levels (Fig. 4*A*). Overall, the 1.3- to 1.4-fold increase in ICAM-1 post-e-cig aerosol inhalation (Fig. 4*B*) implies that the endothelial inflammation that accompanies one single episode of e-cig vaping is not comparable with chronic vascular inflammatory diseases, where a two- to 2.5-fold increase in ICAM-1 in the circulation has been reported (78, 81).

Endothelial activation was monitored by measuring ICAM-1 expression as well as ROS production by human pulmonary endothelial microvascular cells (a human immortalized cell line; HPMVEC clone ST1.6R) that has been used extensively to represent the human lung vascular network (14, 38). ICAM-1 induction on the endothelium is typically a marker of the onset of inflammation. As we reported earlier, pulmonary endothelial cells do not express ICAM (typically showing very low levels of this adhesion molecule), but ICAM-1 is induced in an activated pulmonary endothelium via ROS-mediated signals (55). This increased ICAM-1 expression in HPMVEC posttreatment with serum from subjects pre- and post-e-cig challenge (Fig. 5) indicates that oxidants and other species released into the blood postinhalation induce ICAM-1 expression on the endothelium. This observation also points to the inflammatory burden in the circulating blood that can potentially affect the entire vascular network.

ROS generation is considered to be a marker of endothelial activation, as it represents the first step in the onset of signaling events that lead to the pathogenesis of many vascular disorders (11, 22, 65). ROS was detected in all samples treated with serum after e-cig aerosol inhalation, with the peak ROS generation occurring between 30 and 120 min postvaping and falling off thereafter (Fig. 6). The species of ROS (i.e., H_2O_2 or O_2^-) were not identified. Some of the authors' earlier work has shown that HPMVEC produce O_2^-, but these short-lived radical anions rapidly dismutate into H_2O_2 and O_2·(13, 38). CellROX Green, the dye used, fluoresces upon oxidation and is thus unable to distinguish between the two. ROS production was also observed in cells treated with serum from regular e-cig smokers (Fig. 6*B*). The average ROS values across six e-cig smokers were comparable with that in smoking-naïve individuals exposed to our protocol of e-cig inhalation (Fig. 6*C*, dotted line). This coupled with reduced NO in regular e-cig smokers is indicative of the fact that within the "peak response window," acute e-cig inhalation has the potential to cause vascular dysfunction comparable with that in regular e-smokers.

Earlier investigations by some of the authors using pulmonary microvascular cells had established NADPH oxidase, specifically NADPH oxidase 2, as a major source of ROS production upon inflammatory stimulation of these cells (10, 14). NADPH oxidase 2 activation has also been implicated to play a major role in endothelial dysfunction and vascular disease (22). Our data showing the dampening of ROS production upon pretreatment of HPMVEC with NADPH oxidase 2 inhibitor (apocynin) indicate that activation of NADPH oxidase 2 is the source of ROS production with e-cig serum exposure (Fig. 7).

In conclusion, in our sample of nonsmoking adults, acute e-cig aerosol inhalation was found to lead to a significant increase in oxidative stress and inflammation indices in the serum. It is well known that oxidative stress and inflammation pathways are interconnected in that oxidants can activate proinflammatory transcription factors that lead to production of inflammatory molecules (such as cellular adhesion molecules); conversely, inflammation leads to activation of both endothelial and immune cells which produce ROS, thus causing oxidative stress. Our observation of the transient increase (baseline values were somewhat restored in ~6 h) in markers of both inflammation and oxidative stress with acute exposure to nicotine-free e-cig aerosol points to the potential for e-cigs to pose as health hazard when inhaled, unrelated to nicotine. In addition to the direct effect of e-cig aerosol, the circulating burden of oxidative stress and inflammatory moieties can affect the vascular endothelial network, as outlined in Fig. 8. The NADPH oxidase 2 pathway was key to ROS production by endothelial cells incubated with serum; this pathway is well established to regulate vascular remodeling associated with several cardiovascular pathologies. Its activation suggests that chronic e-cig exposure can potentially trigger signals that drive a vascular pathology phenotype. Elsewhere, the effect of e-cig flavorings alone was reported to cause endothelial oxidative stress (29), although the source of ROS production was not identified. However, structural alterations to the vasculature (myocardial function and arterial stiffness) with acute e-cig challenge have not been noted (71). Long-term repeated e-cig aerosol exposure and the subsequent oxidative stress and inflammatory burden on the circulation could likely cause these changes.

Despite the limitation in the form of small sample size, this study is unique in that repeated blood sampling over several hours provided detailed insight into the time course of the inflammatory and oxidative stress response and pulmonary endothelial activation as a result of the insult caused by the aerosol constituents The typical behavior found indicates a peak response during the 30- to 120-min window and a return to baseline at 6 h of the acute inflammatory and oxidative challenge in this tight group of young healthy nonsmokers. Regular e-cig users showed baseline values of oxidative stress that were similar to smoking-naïve subjects post e-cig inhalation. This indicates the potential for comparable detrimental effects during the peak response window in smoking-naïve subjects as well. Of course, for these subjects there is a return to the pre-inhalation baseline values, so the overall response

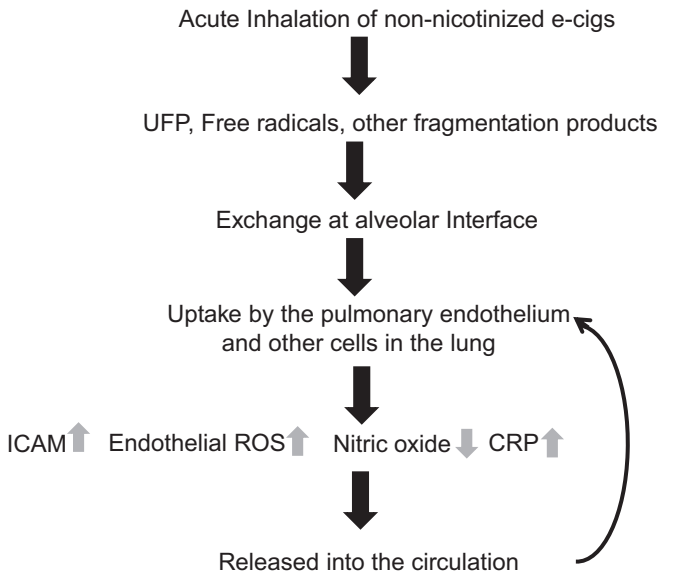


Fig. 8. Schema showing proposed outline of the signaling events post-e-cigarette (e-cig) inhalation. Non-nicotinized e-cig inhalation as an initiator of vascular inflammation and oxidative stress. The detrimental chemical species that are inhaled from e-cig trigger an endothelial signaling pathway that drives inflammation and oxidative stress and also increases the inflammatory burden of the circulating blood.

will likely be different from regular e-smokers for whom repeated inhalation of e-cig aerosol would prevent the recovery to baseline conditions. Such individuals are therefore expected to have elevated levels of the measured inflammation markers at all times during and between ongoing repeated exposures to e-cig aerosol inhalants. Investigations of chronic response, however, are outside the scope of the present investigation, the purpose of which was to determine the kinetics of acute oxidative stress and inflammatory response of e-cig aerosol inhalation in view of a forthcoming study involving assessment of both biological and quantitative MRI markers.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.C. and F.W.W. conceived and designed research; S.C., J.-Q.T., and A.J. performed experiments; S.C. and A.C. analyzed data; S.C., W.G., M.C.L., and F.W.W. interpreted results of experiments; S.C. prepared figures; S.C. drafted manuscript; S.C., M.C.L., and F.W.W. edited and revised manuscript; S.C., J.-Q.T., A.J., W.G., A.C., M.C.L., and F.W.W. approved final version of manuscript.

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