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Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst

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Abstract

E-cigarette flavorings have not been thoroughly evaluated for inhalational toxicity. We have shown that the flavoring chemical cinnamaldehyde impairs human neutrophils, macrophages, and natural killer cells. Here we investigated the effects of other common e-liquid flavoring chemicals on phagocytosis and oxidative burst in neutrophils. We demonstrate that cinnamaldehyde and ethyl vanillin dose-dependently decrease oxidative burst and that benzaldehyde and benzaldehyde propylene glycol acetal dose-dependently impair phagocytosis. Isoamyl acetate did not affect either measure of neutrophil function. These data suggest that inhaling aromatic aldehydic flavoring chemicals, such as cinnamaldehyde, benzaldehyde, benzaldehyde propylene glycol acetal, or ethyl vanillin, could impair neutrophil function.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.9b00171.

Overall study demographics; maximum OCR for each flavoring chemical; time to maximum OCR for each flavoring chemical; maximum ECAR for each flavoring chemical; time to maximum ECAR for each flavoring chemical; IC50 values for neutrophil oxidative burst AUC and phagocytosis; neutrophil oxidative burst rate graphs; neutrophil glycolysis during oxidative burst (PDF)

The authors declare no competing financial interest.

The growing popularity of e-cigarettes has been linked to the public opinion and promotion of them as a safer alternative to tobacco cigarettes¹ and some studies suggesting e-cigarettes help in smoking cessation.² Of particular concern is adolescent use of e-cigarettes. Currently, 20.8% of high school students in the US are e-cigarette users.³ E-cigarettes heat and aerosolize e-liquids, which are typically composed of humectants (propylene glycol (PG) and vegetable glycerin), nicotine, and flavoring chemicals. The wide variety of e-liquid flavors is one of the primary reasons that using e-cigarettes is so appealing to adolescents,^{4,5} yet many of these flavoring chemicals have not been evaluated for inhalational toxicity.⁶ Hence, understanding the potential toxicity of e-cigarette flavorings is necessary to inform regulation of e-liquid manufacturing and to educate the public about e-cigarette safety.

Neutrophils, along with airway macrophages, are one of the body's first lines of defense against inhaled pathogens,⁷ making them an important target for inhaled toxicants. There is emerging appreciation for the complex interplay between cellular metabolism and innate immune response as well as the concept that bioenergetic changes are at the center of innate immune dysfunction and immune cell phenotypes.⁸ However, bioenergetic characterization of the innate immune system has been limited primarily to macrophages, while neutrophils have been largely ignored.^{9,10} Furthermore, the ability of toxicants to alter neutrophil bioenergetics and downstream functions remains unclear. Our lab has demonstrated that the flavoring chemical cinnamaldehyde significantly affects cellular bioenergetics, which in turn causes impairment of key innate immune functions of respiratory epithelial cells.¹¹ Hence, disruption of cellular bioenergetics could be a central mechanism mediating adverse effects of flavoring chemicals.

In this study, we investigated the effects of four common flavoring chemicals, cinnamaldehyde (cinnamon), ethyl vanillin (vanilla), benzaldehyde (almond or cherry), and isoamyl acetate (banana), on neutrophil oxidative burst and phagocytosis. Because we had previously observed impairment of airway and immune cell function by cinnamaldehyde,^{11,12} we were interested in whether other common aromatic aldehydes found in e-cigarettes also impaired cellular function.^{13–15} We chose isoamyl acetate, an ester that is commonly detected in e-cigarettes,¹⁵ for comparison. We also assessed the effects of benzaldehyde PG

acetal, which can form via the reaction of benzaldehyde and PG in e-liquids¹⁶ and has been identified by multiple research groups in e-liquids and e-cigarette aerosols.^{13–15}

We isolated neutrophils from venous blood of healthy human subjects as described previously (Table S1).¹² The effect of flavoring chemicals (Sigma-Aldrich, ST. Louis, MO, USA) on neutrophil oxidative burst was measured via sequential injection of a flavoring compound (0–5 mM final concentration) followed by injection of protein kinase C agonist phorbol 12-myristate 13-acetate (PMA, 100 ng/mL final concentration) on a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) at 37 °C (Figure 1A). Concentrations between 0–5 mM were used because this is approximately two orders of magnitude below maximum concentrations of these flavorings in e-liquids, and we have observed cellular effects on other cell types in this concentration range.^{12–15} The total oxygen consumption during oxidative burst represents the amount of oxygen converted by the neutrophils to superoxide. This was quantified by calculating the area under the curve after PMA stimulation using oxygen consumption rate (OCR, pmol/min) data (Figure 1, Figure S1). Extracellular acidification rate (ECAR, mpH/min), a measure of glycolysis, was simultaneously measured with OCR during these experiments.

Cinnamaldehyde and ethyl vanillin decreased total oxygen consumption in a dose-dependent manner (Figure 1B,C), with a calculated IC₅₀ of 0.39 ± 0.04 mM and 0.42 ± 0.1 mM, respectively. Benzaldehyde and benzaldehyde PG acetal significantly decreased oxygen consumption only at the highest dose, 5 mM (Figure 1D,E). Isoamyl acetate did not affect oxygen consumption during oxidative burst (Figure 1F).

Using the area under the curve analysis, we also obtained the maximum OCR, time to the maximum OCR, and time to baseline OCR. The data for the maximum OCR followed similar trends to those of the total oxygen consumption data (Table S2). The highest doses of ethyl vanillin and benzaldehyde (5 mM) significantly decreased the time to maximum OCR ($p < 0.05$, Table S3). There was no significant difference in the time it took to return to baseline for any of the doses of the flavoring chemicals (data not shown). The ECAR data mirror the OCR data, as expected (Figure S2, Tables S4 and S5).

Next, we determined the effect of e-cigarette flavoring chemicals on neutrophil phagocytosis. Neutrophils plated in a 96-well plate were challenged in triplicate with 0–5 mM of flavoring chemical for 1 h at 37 °C, and phagocytosis was measured using pHrodo Red *Staphylococcus aureus* BioParticles (Thermo Fischer Scientific, Waltham, MA, USA) as described previously.¹² Cinnamaldehyde (CA, 1 mM) was used as a positive control for inhibition of phagocytosis.¹²

We have previously shown that cinnamaldehyde impairs neutrophil phagocytosis.¹² Here, we show that other aromatic aldehydes are also capable of impairing neutrophil phagocytosis. Ethyl vanillin, benzaldehyde, and benzaldehyde PG acetal significantly decreased neutrophil phagocytosis (Figure 2A/B/C), while isoamyl acetate (Figure 2D) had no effect. The inhibition caused by benzaldehyde PG acetal was more potent than that observed with benzaldehyde, with IC₅₀ values of 4.72 ± 2.05 and 1.89 ± 0.66 mM for benzaldehyde and benzaldehyde PG acetal, respectively, suggesting that chemicals formed

via the reaction of flavoring chemicals and e-liquid constituents under ambient conditions may exert stronger toxicological effects than those of the base flavoring chemical.

While there are thousands of different commercially available flavored e-liquids,¹⁷ they are created using a much smaller set of flavoring chemicals that are combined to create unique flavors.^{13–15} Chemical analyses of flavored e-liquids and e-cigarette aerosols from separate studies reveal that many different flavored e-liquids share common flavoring chemicals and that these flavoring chemicals are present in e-liquids at up to molar concentrations.^{13–15} Interestingly, the flavoring chemicals we found to impair neutrophil function share a common chemical class: they are all aromatic aldehydes or their derivatives.

Though they share a common chemical class, these flavorings have unique functional groups, which may explain their differential activities. For example, cinnamaldehyde is an α,β -unsaturated aldehyde that can covalently bind and modify thiols, including interactions with cysteinyl groups on proteins,¹⁸ whereas vanillin (the sister compound to ethyl vanillin) has been shown to interact with proteins via formation of Schiff bases.¹⁹ Benzaldehyde shares structural similarity to cinnamaldehyde but lacks the reactive α,β -unsaturated moiety, which may explain why it inhibited neutrophil function to a lesser extent than cinnamaldehyde. Potential mechanisms underlying decreased oxidative burst observed here could include direct inhibition of key proteins in glycolysis or the pentose phosphate pathway via thiol modification, alteration of glucose uptake, or interference with NADPH oxidase complex formation and activation. The bioenergetic effects of these chemicals could also have implications for assays such as the MTT assay, which relies on mitochondrial metabolism as an indicator of cytotoxicity.

Erythropel et al. recently demonstrated that aldehydic flavoring chemicals such as cinnamaldehyde and benzaldehyde can react with PG under ambient conditions to form flavorant PG acetals,¹⁶ which have been reported in e-liquids and e-cigarette aerosols.^{13–15} Here, we demonstrated that benzaldehyde PG acetal can impair phagocytosis more potently than benzaldehyde (Figure 2B,C). However, benzaldehyde impaired oxidative burst slightly more potently than benzaldehyde PG acetal (Figure 1D,E). These results further support the previously published notion that flavoring chemicals present in e-liquids can also form secondary or tertiary reaction products through interactions with various components of the e-liquid, which alter their biological activities and toxicities.

Among the most challenging components of *in vitro* experimental research models is estimating physiologically relevant doses of inhaled flavoring chemicals. The flavoring chemicals we studied have been reported in e-liquids in hundreds of millimolar to molar concentrations,^{12,14,15,20} and these flavoring chemicals carry over to aerosol with high efficiency.^{16,20} Our highest doses are two orders of magnitude below these ranges; however, because human *in vivo* exposure has not been precisely quantified, it is difficult to compare the doses at which we found effects to the level of exposure in e-cigarette users.

In addition, our results show differing potencies and effects of the flavoring chemicals on oxidative burst and phagocytosis (Table S6). This discrepancy may be due to the method of neutrophil activation used. Uptake of *S. aureus* BioParticles more accurately recapitulates

neutrophil phagocytosis *in vivo*, while stimulation of neutrophils with PMA directly activates protein kinase C, in the absence of a relevant pathogen.

Overall, our data demonstrate that flavoring chemicals present in e-liquids can impair neutrophil function at different levels. Impaired neutrophil function has been shown to play a role in decreased bacterial clearance, especially in vulnerable patients such as those with chronic obstructive pulmonary disease.²¹ Thus, inhalation of e-cigarette flavorings, especially aldehydes, could significantly impair neutrophil function and consequently increase susceptibility to infection and respiratory disease. Since we found that chemicals from the same class (e.g., aromatic aldehydes) may affect immune cell function in a similar manner, assessing the toxicity of flavoring chemicals in e-cigarettes could be done by chemical class rather than by individual compound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CA	cinnamaldehyde
ECAR	extracellular acidification rate
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
OCR	oxygen consumption rate
PG	propylene glycol
PMA	phorbol 12-myristate 13-acetate

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Figure 1.

Neutrophil oxidative burst. (A) Seahorse Extracellular Flux assay was used to measure the effects of flavoring chemicals (B) cinnamaldehyde, (C) ethyl vanillin, (D) benzaldehyde, (E) benzaldehyde PG acetal, (F) and isoamyl acetate on PMA-stimulated neutrophil oxidative burst. Oxygen consumption rate (OCR, pmol/min) was used to determine total oxygen consumption by integrating the oxygen consumption rate (OCR, pmol/min) of PMA-stimulated neutrophils over time (area under the curve, AUC). Data represents mean $\pm$ SEM with $n = 6$.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2.

Neutrophil phagocytosis. Effects of flavoring chemicals (A) ethyl vanillin, (B) benzaldehyde, (C) benzaldehyde PG acetal, and (D) isoamyl acetate on neutrophil phagocytosis as assessed with pHrodo red *S. aureus* BioParticles. Data represent mean ± SEM with $n = 6$, with the exception of $n = 3$ for 5 mM isoamyl acetate.

* $p < 0.05$, ** $p < 0.01$.