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Oxidative stress-mediated EGFR activation by cigarette smoke or heated tobacco aerosol in human primary bronchial epithelial cells from multiple donors

Short title: EGFR activation by cigarette smoke or heated tobacco aerosol exposure

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Abstract

The epidermal growth factor receptor (EGFR) signaling pathway has essential roles in maintaining homeostasis of various tissues by regulating cell proliferation and differentiation. Deregulation of the EGFR signaling pathway is associated with various chronic diseases including chronic obstructive pulmonary disease. Cigarette smoke (CS) is known to activate EGFR, which is linked to chronic obstructive pulmonary disease. The biological sequence from CS exposure to EGFR activation is initiated by oxidative stress caused by intracellular reactive oxygen species (ROS) and the depletion of glutathione, which led to EGFR ligand secretion and EGFR activation. We hypothesized that reducing exposure to CS constituents contributes

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to preventing CS-inducible EGFR activation. Therefore, we examined the aerosol from heated tobacco products (HTPs) because the aerosol contains fewer chemical constituents at lower levels than CS. We exposed primary human bronchial epithelial cells from four donors to the extracts of CS from a 1R6F reference cigarette or HTP aerosol from three in-market products, including our DT3.0a. The biological sequence from ROS to EGFR activation were assessed. CS induced all the tested endpoints although inter-donor differences were observed, whereas HTPs elicited most of the biological events at higher concentrations; however, EGFR phosphorylation was not observed even at five-fold higher concentration than CS. Overall our results indicate that HTPs are less effective than CS to elicit ROS-induced EGFR activation. The reduced-risk potential of HTPs on EGFR-related diseases should be investigated further. In addition, testing with multiple donors is warranted when considering the individual differences in responses of primary cells to stimuli.

Short abstract

We examined a biological sequence leading to EGFR activation by exposing bronchial epithelial cells from four different donors to cigarette smoke (CS) or aerosol from three in-market heated tobacco products (HTPs). CS and HTP aerosols elicited oxidative stress and EGF ligand secretion, whereas higher concentrations of HTPs than CS were required for the same effects. EGFR activation was only observed after CS exposure. This is the first step in investigating the reduced-risk potential of HTPs in EGFR signaling-mediated adverse outcomes.

Introduction

Epidermal growth factor receptor (EGFR), which is a member of the tyrosine kinase family, has key roles in tissue homeostasis by regulating cell proliferation and differentiation. Hundreds of genes are regulated by the EGFR signaling pathway, and the proper regulation of these gene expression levels is critical to maintaining tissue functions (Chen et al., 2016). The overexpression and abnormal activation of EGFR are associated with the malfunction of various tissues and organs such as the skin, kidney, and lung (Harskamp et al., 2016; Holcmann & Sibilia, 2015; Vallath et al., 2014). EGF, heparin binding-EGF-like growth factor (HB-EGF), transforming growth factor alpha (TGF-α), and amphiregulin (AREG) are EGFR ligands that mediate their effects after binding via the autophosphorylation of EGFR, and then transduce the downstream signaling pathway to express EGFR-regulated genes (Singh et al., 2016).

Luettich et al. proposed an EGFR-mediated simplistic biological pathway that led to a decrease in lung function (i.e. adverse outcome pathway for “lung function decrease”), which is elicited by reactive oxygen species (ROS) (Luettich et al., 2017). ROS, as well as oxidative stress, has been suggested to induce the expression and secretion of EGFR ligands, which activate EGFR (Miyazaki et al., 1996) in a ligand-dependent manner. Of note, EGFR is also

activated via a ligand-independent mechanism (Paulsen et al., 2011; Shao & Nadel, 2005; Sheng et al., 2020). EGFR has prominent functions, including tissue development and repair in the airway epithelium. Furthermore, the activation of EGFR leads to the expression of mucins and goblet cell metaplasia, which are features seen in hypersecretory diseases of the airways (Casalino-Matsuda et al., 2006).

One of known causes of lung disease is cigarette smoking. Cigarette smoke (CS) contains thousands of chemicals, some of which can elicit intracellular ROS and oxidative stress. In addition, Khan et al. reported that CS exposure elicited EGFR activation (Khan, Lanir, Danielson, & Goldkorn, 2008). Therefore, oxidative stress-mediated pathogenesis of lung diseases might be avoidable by reducing exposure to such chemical substances. The US FDA has proposed guidance for assessing the harm reduction potential of a "modified risk tobacco product" (MRTP) based on the possibility that decreased toxicant exposure may lead to health benefits (U.S. Food and Drug Administration, 2012). One of the expected MRTPs is heated tobacco products (HTPs), which generate vapor by heating but not combusting tobacco leaves; thus, the aerosol contains lower levels of harmful constituents than CS (Hirn et al., 2020; Poget et al., 2021). Several previous studies showed that the aerosol of HTP elicited less biological effects in *in vitro* and *in vivo* studies, as well as clinical testing (Ito et al., 2019; Phillips et al., 2016; Thorne et al., 2020; Yuki et al., 2022). Moreover, the FDA has approved one HTP as an MRTP (i.e., reduced-exposure product) after rigorous examination of scientific evidence not limited to exposure reduction, but also the reduction of biological impact and expectation of health benefit for the population as a whole (U.S. Food and Drug Adnimistration, 2020). Nevertheless, further evidence that augments our understanding regarding emissions from HTPs show that reduced biological responses when compared with those of CS is required because CS elicits a wide range of biological effects. Although clinical and epidemiological studies have provided the most relevant data regarding the health effects in humans, *in vitro* studies using human cells might help infer the potential toxicity and underlying mechanisms of test products in humans and uncover their associated mechanisms (Gohlsch et al., 2019). Although HTPs induced lower levels of oxidative stress responses than cigarettes (Munakata et al., 2018; Taylor et al., 2018), the effects on the downstream events of oxidative stress after HTP exposure remain unclear. An evaluation of the sequence of biological events from oxidative stress to EGFR activation would thus be beneficial for assessing the reduced risk potential of HTPs.

The application of *in vitro* tests for risk assessment is in line with the recent growing trend toward the reduction, replacement, and refinement (3R principles) of animal testing. An AOP framework is such a methodology, which depicts simplistic biological pathways leading to adverse outcomes based on accumulated knowledge. Thus, AOP-based risk assessment may provide mechanistic reasoning for toxicity and risk. In this study, we partially applied the AOP proposed by Luettich et al., in which the molecular initiating event is intracellular ROS

(Luettich et al., 2017). Although the adverse outcome of the proposed AOP is "lung function decrease", we focused on the biological sequence from ROS to elicitation of EGFR activation because the biological events are related to various lung diseases as mentioned above.

To investigate the reduced risk potential of HTPs using the AOP framework, we exposed the aerosol collected mass (ACM) of our proprietary Direct heating Tobacco System Platform 3 Generation 3 version a (DT3.0a), and other in-market HTPs, and the total particulate matter (TPM) of combustible cigarettes. This information should help us understand the reduced risk potential of HTPs.

Materials and methods

Test products

Three commercially available HTP products were used in this study: DT3.0a, tobacco heating system (THS), and tobacco heating products (THP). All of these HTPs were purchased from the Japanese market. The THS heats tobacco stick directly via a heating blade inside the system, whereas THP heats tobacco stick peripherally. DT3.0a employs peripheral heating with convection flow. The heating temperature and the result of representative chemical analysis were reported previously (Eaton et al., 2018; Forster et al., 2018; Jaccard et al., 2017; Mallock et al., 2019). A regular tobacco-flavored heating stick for each product was used. The 1R6F reference cigarette (University of Kentucky, Lexington, KY USA) was used as a representative conventional cigarette.

Preparation of cigarette smoke total particulate matter and heated tobacco aerosol collected mass

CS-TPM and HTPs-ACM were prepared in accordance with the International Organization for Standardization Intense smoking regimen (a 55 mL puff taken over 2 seconds, repeated every 30 seconds) (ISO 20778, 2018). The ventilation holes of the 1R6F were blocked and it was then applied to a Borgwaldt RM20H smoking machine. The TPM was collected on a 45-mm diameter Cambridge filter pad and extracted using dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

HTPs-ACM was generated in the same manner as for the 1R6F cigarettes with a LM5E smoking machine (Borgwaldt) but without vent blocking (ISO 20778, 2018). The initial concentrations of CS-TPM and HTPs-ACM were adjusted to 40 and 200 mg/mL, respectively, with DMSO. The CS-TPM and HTPs-ACM in DMSO solution were then immediately aliquoted for single use and stored at −80°C until use. Each aliquot was thawed just before each medium change and diluted with the culture medium for exposure.

Nicotine concentrations in CS-TPM and HTPs-ACM

The nicotine concentration in each test product was determined by gas chromatography using an Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) with flame ionization detection. The limit of detection and limit of quantification were 12.58 μg/mL and 42 μg/mL, respectively.

Cell culture and exposure conditions

Primary Normal Human Bronchial Epithelial (NHBE) cells derived from four different donors were purchased from LONZA and cultured in Airway Epithelial Cell Growth medium (PromoCell, Germany) supplemented with growth factors at 37°C in a 5% CO_2 atmosphere. Donor information is summarized in Table 1. Exposure to the test products was conducted

under the same conditions and untreated cells were considered controls.

The maximum concentration of each test substance was selected to not exceed 1% DMSO. These maximum concentrations of TPM and ACMs were 400 and 2000 μg/mL, respectively. We confirmed that exposure to the TPM or ACM at the highest concentration did not induce severe cytotoxicity (Figure S1) in a 1 hour exposure.

ROS assay

Intracellular ROS production was determined using the non-fluorescent compound CM-H2DCFDA (Invitrogen Corporation, Carlsbad, CA, USA): 96-well cell culture plates were initially seeded with ~~1×10^4~~-10,000 NHBE cells per well followed by 24 h incubation at 37°C. NHBE cells were incubated with 5 μM CM-H2DCFDA, 1 μM Hoechst 33342 (Dojindo Laboratories), and 1 μM Celltracker Red (Invitrogen Corporation) of HBSS containing 0.5% FBS for 30 min. After washing, NHBE cells were exposed to the test product CS-TPM or HTPs-ACM for 1 h. Fluorescence images were taken and captured by an Operetta CLS and Harmony software (PerkinElmer Cellular Technologies Germany GmbH, Hamburg, Germany). To eliminate the autofluorescence of CS-TPM and HTPs-ACM, background fluorescence values with no probe were subtracted from the relative fluorescence units (RFU) with a probe, normalized to the vehicle control (1% DMSO) for all cases, and expressed as fold change in assay signal. The experiment was performed on three independent occasions per donor.

Glutathione assay

The glutathione level was determined using a monochlorobimane (MCB) (Sigma–Aldrich) dye: 96-well cell culture plates were initially seeded with 10,000 NHBE cells per well followed by 24 h incubation at 37°C. NHBE cells were exposed to the test products CS-TPM or HTPs-ACM for 1 h. After washing, NHBE cells were stained with 200 μM MCB, 10 μM Syto 13, and 1 μM Celltracker Red for 30 min. The fluorescence images were taken and captured by an Operetta CLS and Harmony software (PerkinElmer). To eliminate the autofluorescence of CS-TPM and HTPs-ACM, background fluorescence values with no probe were subtracted from the RFU with a probe, normalized to the vehicle control (1% DMSO) for all cases, and expressed as fold change in assay signal. The experiment was performed on three independent occasions per donor.

Measurement of EGFR ligands

First, 96-well cell culture plates were seeded with 10,000 NHBE cells per well followed by 24 h incubation at 37°C, then exposed to the test products CS-TPM and HTPs-ACM for 1 h. The supernatants of the NHBE cells were collected and the concentrations of TGF-α and amphiregulin (AREG) were determined by a Luminex assay (R&D Systems) using the Bio-plex 200 (Bio-Rad, Hercules, CA, USA) and a Quantikine Human Amphiregulin ELISA Kit

(R&D Systems), respectively. The experiment was performed on three independent occasions per donor.

Measurement of phosphorylated EGFR

First, 48-well cell culture plates were seeded with 30,000 NHBE cells per well followed by 24 h incubation at 37°C, and then exposed to the test products CS-TPM and HTPs-ACMs for 1 h. Cells were washed with ice-cold HBSS before extraction with 100 µL cell lysis buffer. Phosphorylation of EGFR was measured using a sandwich ELISA with phospho-specific EGFR (pY1068) antibodies (AlphaLISA SureFire p-EGF Receptor (Tyr1068) Assay Kit, PerkinElmer) following the manufacturer's instructions. The protein concentration of lysates was determined by a Micro BCA assay (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The signal intensity of pEGFR was then normalized to the protein concentration. Cytation 5 (Agilent Technologies) was used to determine pEGFR and protein concentrations. The experiment was performed on three independent occasions per donor.

Because the culture medium contained 10 ng/mL EGF as a supplement, the dynamic range of EGFR phosphorylation was also examined by adding 20 ng/mL EGF to the culture medium for 1 h. As a result, we confirmed approximately 4-fold induction of EGFR phosphorylation (Figure S2).

Calculation of a benchmark dose

The results of a dose response for each assay were analyzed by benchmark dose (BMD) modeling using the U.S. Environmental Protection Agency (EPA) Benchmark Dose Software (BMDS, version 3.2) in accordance with the BMD technical guidance (U.S. Environmental Protection Agency, 2012). We used BMD to quantitatively compare the biological responses between cigarettes and HTPs rather than to calculate points of departure to determine a human equivalent dose for risk assessment. The best fit model was selected among the suite of continuous models based on BMDS recommendations from decision logic criteria (i.e., the model with the lowest Akaike's Information Criterion (AIC) or lowest BMDL). The BMDL refers to the two-sided (upper and lower) bounds of the 95% confidence intervals of the BMD. Then, the BMD values were calculated using the selected model to describe changes in response corresponding to the background of one standard deviation (SD) of the mean of the control value. This setting was applied throughout this study, because use of one SD is recommended by U.S. EPA for comparison purposes (U.S. Environmental Protection Agency, 2012).

To interpret the results conservatively, some donors that did not show a response were eliminated from the calculation in this study. The data calculated with all donors are shown in the supplemental information (Table S1).

We assumed dose-dependent increases in ROS, EGF ligands, and EGFR activation, and a dose-

dependent decrease in GSH. The BMD calculation was not performed when a clear dose-dependent change was not observed.

Statistical analysis

Dunnett's multiple comparison was performed to examine the statistically significant difference between CS-TPM and each HTP-ACM at the comparable concentration of 400 μg/mL, using the pooled donor data. A p-value less than 0.05 was considered statistically significant.

Results

Nicotine concentrations in test products

We quantified the nicotine concentrations in the undiluted CS-TPM and HTPs-ACM. Table 2 shows the CS-TPM and HTPs-ACM concentrations and their nicotine concentrations. NHBE cells were exposed to CS-TPM and HTPs-ACM at different concentration ranges: CS-TPM (0–400 μg/mL) and HTPs-ACM (0–2000 μg/mL). The concentrations of nicotine at the highest doses were approximately three times higher in HTPs-ACM than in CS-TPM.

Oxidative stress responses

The changes of intracellular ROS and antioxidant glutathione following 1 h exposure to each HTP aerosol were evaluated as indices of the oxidative stress response. CS-TPM and HTPs-ACM exhibited dose-dependent responses in ROS production and GSH depletion, in a consistent manner between the donors (Figure 1 A-D and Figure 2 A-D). Statistically significant differences in ROS and GSH between the comparable concentration of CS-TPM and each HTP-ACM (400 μg/mL) were observed (p-value < 0.0001). A dose response using the pooled donor data was subjected to BMD modeling to compare the responses between cigarettes and HTPs. The calculated BMD (μg/mL) values were consistently higher for HTPs than CS, and no significant differences between HTPs were detected.

Secretion of EGF ligands

Levels of epidermal growth factor ligands, TGF-α and AREG, secreted into the cell culture medium were measured after exposure to all tested products for 1 h. The concentrations of TGF-α and AREG were substantially higher after exposure to CS-TPM than HTPs-ACM for Donors A, B, and D. Significant changes in the EGF ligands were not observed for Donor C after CS-TPM and HTPs-ACM exposure (Figure 3 and 4). A statistically significant difference was observed between the comparable concentration of CS-TPM and each HTP-ACM (400 μg/mL) in TGF-α secretion (p-value < 0.0001), while that in AREG was not statistically significant. The calculated BMD (μg/mL) values were consistently higher in HTPs than CS.

because of the weak dose-response of AREG secretion by HTPs-ACM exposure, the BMD values were generally greater than the highest concentration of each test product (Table 3). To interpret the results conservatively, we eliminated Donor C from the calculation of these events. The results of all donors are shown in the supplementary figure (Figure. S1-3.).

Phosphorylation of EGFR

Levels of phosphorylated EGFR were measured after exposure to all tested products for 1 h. CS-TPM showed dose-dependent responses related to phosphorylated EGFR levels (Figure 5). However, none of the HTPs-ACM increase the phosphorylated EGFR levels. A statistically significant difference in EGFR phosphorylation between the comparable concentration of CS-TPM and each HTP-ACM (400 μg/mL) were observed (p-value < 0.0001). The BMD calculation was therefore not performed for HTPs-ACM. The magnitude of EGFR phosphorylation varied between donors, from approximately 1.2 to 3.5-fold increases at the highest concentration of CS-TPM. Donor A did not elicit significant phosphorylation of EGFR even after CS-TPM exposure. To interpret the results conservatively, Donor A was excluded from the calculations in this event. The calculated BMD (μg/mL) value of 1R6F without Donor A for phosphorylated EGFR was 320.2 (Table 3). The results of all donors, including the eliminated donor, are shown in the supplementary figure (Figure S1-3).

Discussion

This study assessed the sequence of biological responses from oxidative stress to EGFR activation (i.e., phosphorylation) based on the AOP proposed by Luettich (Luettich et al., 2017) in four different NHBE donors and compared them with ACMs from three commercially available HTPs (DT3.0a, THP, and THS) and the TPM from 1R6F reference cigarettes. EGFR activation is a molecular initiating event for some lung diseases, and might be an indicator of the potential risk of developing these diseases. Exposure concentrations of HTPs-ACM were selected up to five times higher than that of CS-TPM because HTPs were reported to contain lower levels of harmful and potentially harmful constituents (HPHCs); thus, we expected that higher concentrations of HTPs-ACM would be required to observe biological responses (Margham et al., 2016; Munakata et al., 2018; Sakaguchi et al., 2014; Schaller et al., 2016). The concentrations of HTPs used in this study achieved up to three times higher nicotine levels than CS-TPM.

As expected, CS induced oxidative stress-related endpoints and their ~~hypothetical~~ downstream events, EGFR ligand expression, and EGFR activation. HTP-ACMs elicited only oxidative stress (i.e., increased ROS and GSH depletion), the uppermost event of the biological sequence that we tested, whereas higher concentrations were needed to obtain similar response

levels after CS-TPM exposure. The occurrence of downstream biological events was weaker or negligible when compared with CS-TPM at the same concentrations. Therefore, this suggested that HTP-ACMs have a limited impact on activation of the EGFR signaling pathway.

Although most of the results in our study were consistent, donor-to-donor variation was observed. Donor C did not elicit EGFR ligand secretion after CS-TPM and HTP-ACMs exposure (Figure 3C and 4C). EGFR ligand secretion is regulated by their expression and shedding by proteinases such as ADAM-17 (Blobel, 2005; Kasina, Scherle, Hall, & Macoska, 2009). Therefore, donor-to-donor variation and lacking responsiveness in this study may be dependent on the activity of such proteinases. However, Donor C showed a marked increase in EGFR phosphorylation (Figure 5C). This implies that CS also induces ligand-independent phosphorylation of EGFR or secretion of other EGF ligands. In addition, Donor A did not elicit significant EGFR activation even when there was an increase in EGFR ligand secretion. These results suggest donor-dependent differences in the threshold of EGFR activation via ligand binding, time-course of ligand binding to receptor activation, or ligand independent phosphorylation (Filosto et al., 2012). The variations observed in primary cell cultures might be explained by the original characteristics of the donors (Bovard et al., 2020; Mori et al., 2022; Rayner et al., 2019). Theoretically, not all individuals have the same transcriptomic, proteomic, and epigenetic characteristics (Jackson et al., 2020; Stefanowicz et al., 2012); therefore, the capacity of the antioxidant system might also vary. Such variations are considered to be the cause of differences in the occurrence of oxidative stress-associated biological events. For proper toxicological assessments, multiple donors are warranted because the use of a non-responsive donor might cause misinterpretation of the toxicological results.

We calculated the BMD values of each endpoint to quantitatively compare the biological responses between cigarette smoke and HTP aerosol. The BMD values of HTPs were much higher than those of cigarette smoke for all endpoints other than EGFR activation. BMD values of HTPs in EGFR activation were not able to calculated due to lack of dose- response. Therefore, it was considered that HTP aerosol induces weaker biological responses for the selected endpoints examined. We also observed that the BMD values of CS-exposed group tended to be high in the order of oxidative stress, EGFR ligand secretion, and phosphorylation of EGFR. Because these endpoints are considered to occur sequentially, BMD values are reasonably expected to increase as the biological response progresses. For a conservative interpretation of the results, we excluded Donors A and C from the BMD calculation of EGFR phosphorylation and EGFR ligand secretion, respectively. However, the inclusion of all donors resulted in a slight variation in BMD values for each event, but the trend did not change significantly (Table S1). As mentioned previously, consideration of donor-to-donor variability is crucial to interpret toxicological results, and the inclusion of additional donors in this study could have resulted in different values of BMD. Although BMD values were consistently higher in HTP aerosol than CS, interpretation of the quantitative difference between them

should be conducted carefully. Furthermore, understanding the difference between in vitro and human in vivo doses is important to interpret the toxicity in human-use scenarios. Esther et al. suggested that the estimated nicotine exposure level in the airways of smokers is 70–850 ng/mL (Esther et al., 2023). This is sufficiently lower than the dose range of this study. However, further investigations are warranted to compensate for the difference.

Overall, our results are in line with previous reports that the effective concentrations of HTPs were higher than those of combustible cigarettes for various toxicological endpoints (van der Toorn et al., 2015; Walczak et al., 2020). ROS is a primary cause of EGFR activation induced by CS exposure. Therefore, reduction of the causative substances of ROS may be important. The causative substances of ROS include phenolic compounds, aromatic hydrocarbons, and metals (Caliri, Tommasi, & Besaratinia, 2021), which are present in HTP aerosol at low levels (Hirn et al., 2020; Poget et al., 2021, Hashizume et al., 2023). Additionally, our preliminary experiment showed that deterioration of ROS by antioxidant treatment (i.e., N-acetyl-cysteine) effectively reduced EGFR activation by CS (Figure S4). Therefore, the reduction and elimination of ROS are also considered to be effective to prevent EGFR activation.

EGFR activation is known to be associated with mucin overexpression, which is often seen in COPD and asthma patients (Burgel & Nadel, 2008; Takeyama et al., 1999). EGFR and its signaling pathway are therefore therapeutic targets for lung diseases (Lai & Rogers, 2010; Vallath, Hynds, Succony, Janes, & Giangreco, 2014). Taken together, prevention of EGFR activation may contribute to reducing the risk. Although further investigations are necessary to draw a conclusion, our results suggest a reduced risk potential of HTPs on EGFR-related diseases.

Bentley et al. reported that most chemical constituents in HTP aerosol (i.e., THS2.2) overlapped with those in cigarette smoke, and only a few chemicals were present at higher levels than in CS (Bentley et al., 2020). This type of study will help us understand the reduced-risk potential and toxicity of HTP aerosol. Taken together with the *in vitro* test results of HTPs reported to date, high levels of chemical constituents in HTP aerosol compared with cigarette smoke are thought to not have acute effects on elicitation of oxidative stress and inflammation. However, limited studies have reported the potential risk of chronic exposure to HTP aerosol, and therefore the effects of chronic exposure to such aerosol constituents remain unclear. The advancement of *in vitro* cell culture platforms enable us to perform repeated exposure study, could thus help to investigate effects of HTP aerosol in chronic exposure (Bovard et al., 2020; Dye et al., 2015).

We demonstrated the reduced risk potential of HTPs in a single exposure of NHBE to aerosol based on some of the AOP proposed by Luettich et al. However, enhancement of the capability of *in vitro* testing with chronic exposure enabled us to investigate the remaining biological sequence to approximate adverse outcomes or disease. Therefore, investigation of the chronic effects of exposure to HTP aerosol might provide deeper insights into its health effects.

Moreover, although we used the BMD calculation to infer the quantitative difference between HTPs and CS, we can improve the link between the results and real-world situations. The combination of *in vitro* studies and *in silico* analyses, such as mathematical modeling of AOP (i.e., quantitative AOP) and simulating aerosol deposition in real-world use scenarios, will be a future direction.

Competing interests

This work involved DT3.0a (PloomX, manufactured by Japan Tobacco Inc.). All authors are employees of Japan Tobacco Inc.

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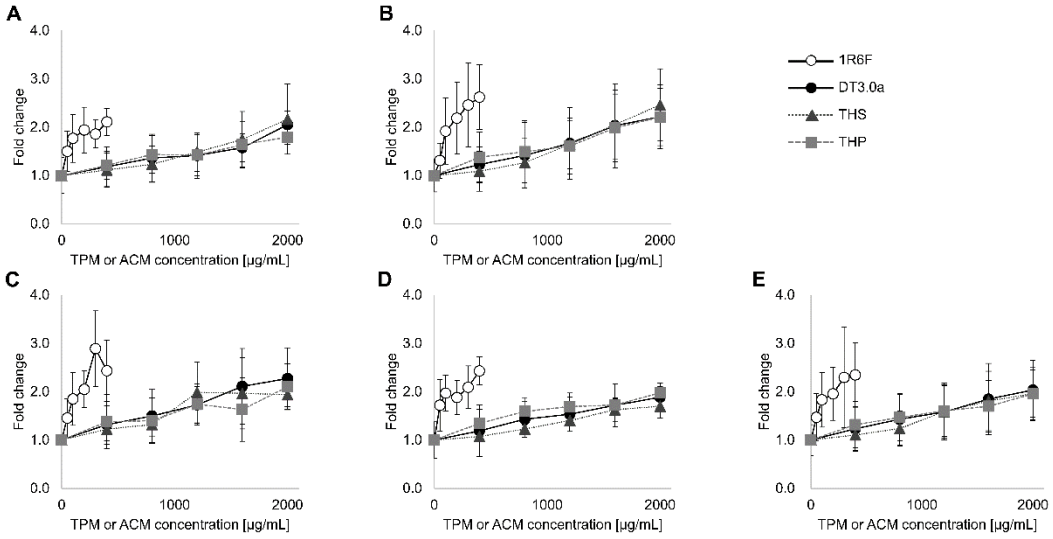

Figure 1. Intracellular ROS in each donor NHBE exposed to CS-TPM or HTPs-ACM. Fold changes in intracellular ROS generation were calculated as a ratio to the non-treatment control of each donor (A-D, Donor A through Donor D). The data are the means and standard deviations of triplicate measurements. Each fold change was then summarized as an all-donor result (E). NHBE; normal human bronchial epithelial cells, ROS; reactive oxygen species, CS; cigarette smoke, TPM; total particulate matter, HTP; heated tobacco product, ACM; aerosol collected mass.

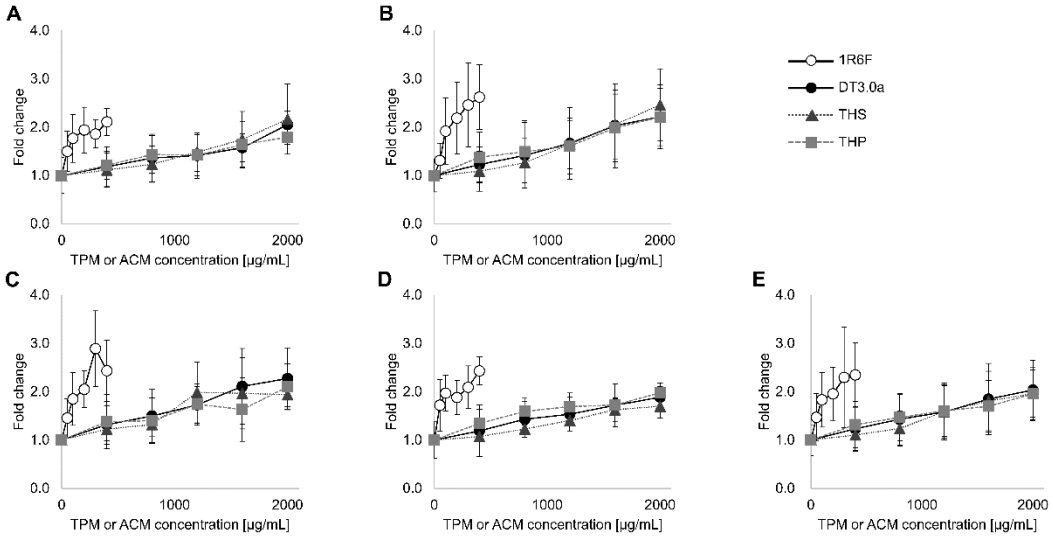

Figure 2. Glutathione depletion in each donor NHBE exposed to CS-TPM or HTPs-ACM. Fold changes in glutathione depletion were calculated as a ratio to the non-treatment control of each donor (A-D, Donor A through Donor D). The data are the means and standard deviations of triplicate measurements. Each fold change was then summarized as an all-donor result (E). NHBE; normal human bronchial epithelial cells, CS; cigarette smoke, TPM; total particulate matter, HTP; heated tobacco product, ACM; aerosol collected mass.

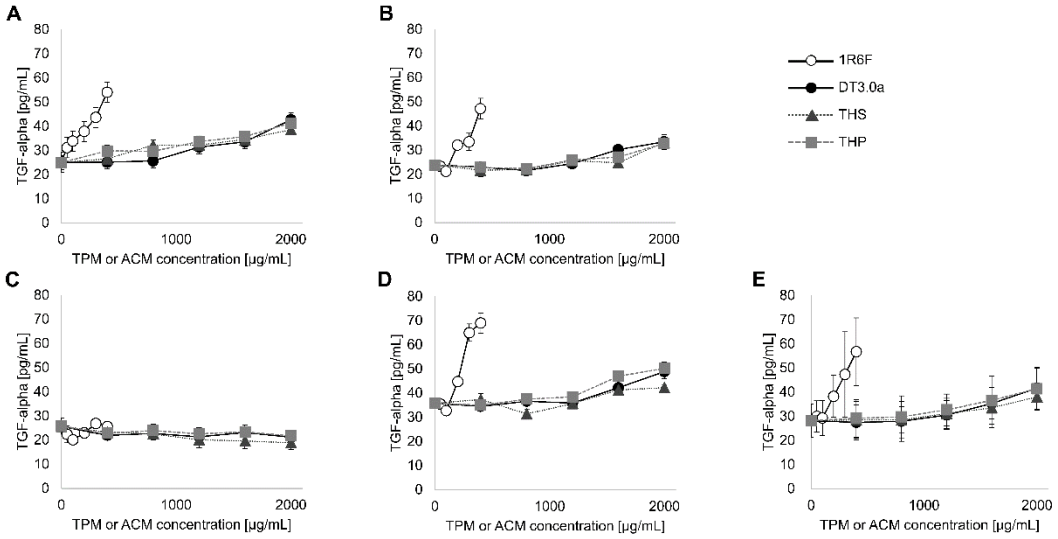

Figure 3. Secretion of TGF-α in each NHBE donor exposed to CS-TPM or HTPs-ACM. The data are the means and standard deviations of triplicate measurements (A-D, Donor A through Donor D). Each fold change was then summarized as an all-donor result (E). Donor C was eliminated from the calculation because the donor cells did not respond to the exposure. TGF-α, transforming growth factor-alpha, NHBE; normal human bronchial epithelial cells, CS; cigarette smoke, TPM; total particulate matter, HTP; heated tobacco product, ACM; aerosol collected mass.

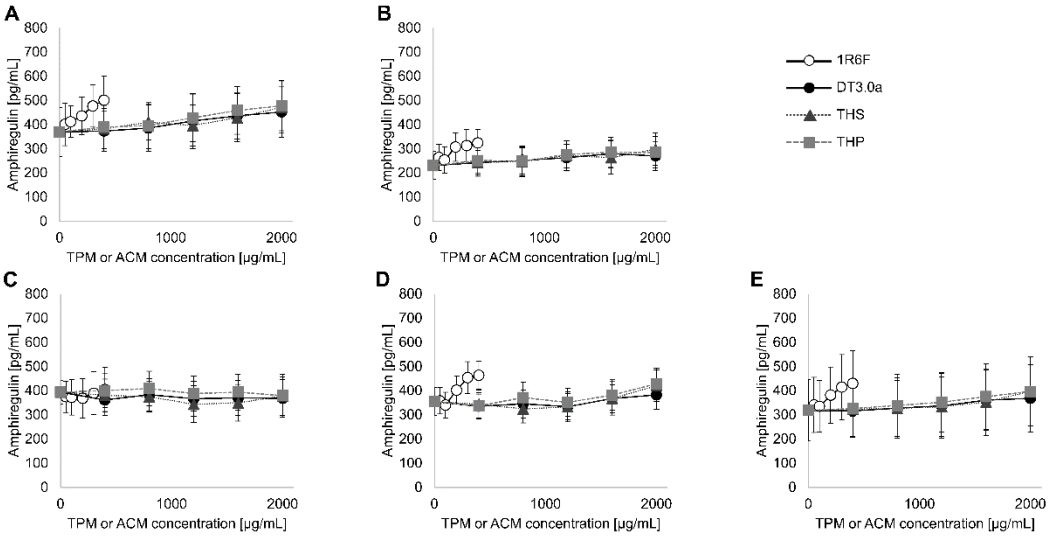

Figure 4. Secretion of AREG in each NHBE donor exposed to CS-TPM or HTPs-ACM. (A) Donor A, (B) Donor B, (C) Donor C, (D) Donor D, and (E) all donors. The data are the means and standard deviations of triplicate measurements (A-D, Donor A through Donor D). Each fold change was then summarized as an all-donor result (E). Donor C was eliminated from the calculation because the donor cells did not respond to the exposure. AREG, amphiregulin, NHBE; normal human bronchial epithelial cells, CS; cigarette smoke, TPM; total particulate matter, HTP; heated tobacco product, ACM; aerosol collected mass.

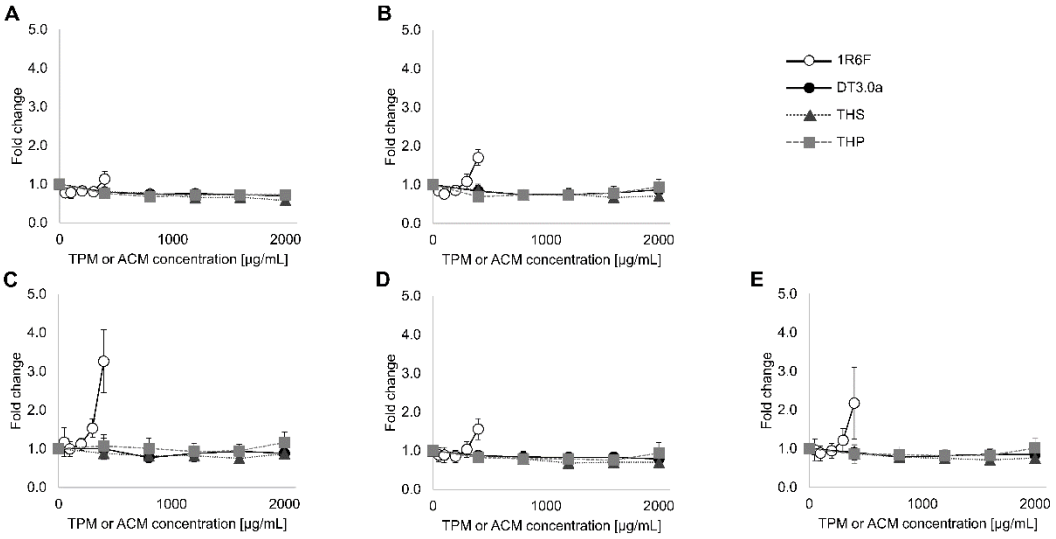

Figure 5. Phosphorylation of EGFR in each NHBE donor exposed to CS-TPM or HTPs-ACM. Fold changes of EGFR phosphorylation levels were calculated as a ratio to the non-treatment control of each donor. The data are the means and standard deviations of triplicate measurements (A-D, Donor A through Donor D). Each fold change was then summarized as an all-donor result (E). Donor A was eliminated from the calculation because the donor cells did not respond to the exposure. EGFR; epidermal growth factor receptor, NHBE; normal human bronchial epithelial cells, CS; cigarette smoke, TPM; total particulate matter, HTP; heated tobacco product, ACM; aerosol collected mass.

Table 1. Donor information.

Donor	A	B	C	D
Age	62	57	50	73
Sex	Female	Male	Male	Female
Race	AA	C	H	AA
Smoker	No	No	No	No

AA, African American; C, Caucasian; H, Hispanic.

Table 2. Concentrations of TPM, ACM, and nicotine in diluted TPM and ACM samples for biological assays.

	1R6F	DT3.0a	THS	HTP
TPM (μg/mL)	Nicotine (μg/mL)			
0	0.00 ± 0.00	-	-	-
50	2.60 ± 0.01	-	-	-
100	5.20 ± 0.02	-	-	-
200	10.40 ± 0.05	-	-	-
300	15.60 ± 0.08	-	-	-
400	20.80 ± 0.10	7.76 ± 0.48	8.00 ± 0.64	6.74 ± 0.10
800	-	15.52 ± 0.97	16.01 ± 1.28	13.48 ± 0.20
1200	-	31.03 ± 1.93	32.02 ± 2.55	26.95 ± 0.40
1600	-	46.55 ± 2.90	48.03 ± 3.83	40.43 ± 0.60
2000	-	62.07 ± 3.87	64.03 ± 5.11	53.90 ± 0.80

1R6F, Kentucky reference cigarette; DT3.0a, Direct heating Tobacco System Platform 3 Generation 3 version a; THS, tobacco heating system; THP, tobacco heating products.

Table 3. BMD (μg/mL) for each endpoint.

	BMD (μg/mL)				
	ROS	GSH	TGF-α	AREG	p-EGFR
1R6F	61.7	41.2	175.2	399.4	320.2
DT3.0a	1049.0	1937.9	1570.6	3194.8*	N/A**
THS	985.5	1535.9	1694.8	2339.1*	N/A**
THP	1129.0	863.0	1531.4	2885.9*	N/A**

*The values were greater than the maximum tested concentration because of the weak dose-response. ** BMD of p-EGFR for HTPs were not calculated because we did not observe a clear dose-dependent increase. 1R6F, Kentucky reference cigarette; DT3.0a, Direct heating Tobacco System Platform 3 Generation 3 version a; THS, tobacco heating system; THP, tobacco heating products; ROS, reactive oxygen species; GSH, glutathione-SH; AREG, amphiregulin; p-EGFR, phosphorylated epidermal growth factor receptor; N/A, not applicable.