

Air pollutants cause release of hydrogen peroxide and interleukin-8 in a human primary nasal tissue culture model

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Background: A component of primary innate defense of the nasal mucosa against inhaled pathogens includes continuous, low-level release of hydrogen peroxide (H₂O₂) into luminal secretions. Epidemiologically, an association exists between poor air quality and increased prevalence of sinonasal disease. To understand the effects of particulate matter (PM) in nasal mucosa, we studied the release of H₂O₂ and interleukin 8 (IL-8) after PM exposure.

Methods: Human nasal specimens were collected from surgery and cultured in serum-free growth medium. Cell integrity and recovery during culture was monitored by lactate dehydrogenase (LDH) release into the medium. Cultures were exposed to PM for 24 hours in the presence/absence of diphenyliodonium sulfate (DPI; a nicotinamide adenine dinucleotide phosphate [NADPH] oxidase inhibitor). Luminex cytokine and Amplex-Red H₂O₂ assays were performed.

Results: LDH levels dropped rapidly within 2 days, indicative of stabilization and cell recovery after harvest. All cultures released H₂O₂ into the medium. Exposure to PM (20 µg/cm²) increased H₂O₂ levels significantly (94.6 ± 7.7 nM) compared to untreated controls (55.8 ± 4.0 nM; *p* = 0.001). PM-induced H₂O₂ production was partially

inhibited by DPI (80.1 ± 3.8 nM), indicating that cellular NADPH oxidase may be a primary source of H₂O₂ production. Exposure to PM increased IL-8 levels in a dose-dependent fashion (control = 2301 ± 412 MFI; 20 µg/cm² = 5002 ± 1327 MFI; 40 µg/cm² = 8219 ± 1090 MFI; *p* = 0.022).

Conclusion: PM increases the quantity of H₂O₂ released by nasal epithelial cells, indicating that PM can contribute to oxidative stress in part by activating a normal cellular defense mechanism. Exposure to PM resulted in elevated IL-8 levels and mucin production in explants. Efforts to reduce airborne PM may lead to reduced H₂O₂ and mucin production in sinonasal epithelium. © 2014 ARS-AAOA, LLC.

Key Words:

PM10; air pollution; rhinosinusitis; IL-8; hydrogen peroxide; nasal tissue culture; NADPH oxidase; DUOX

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Innate immunity refers to a built-in defense system of the host to resist the invasion of pathogens or noxious agents, and the pattern recognition of microbes and other threats by an innate immune system is considered to play a key mechanism in the maintenance of healthy airway surface liquid (ASL).¹ Among the various components that comprise innate epithelial host defense, the continuous release of hydrogen peroxide (H₂O₂) by airway epithelial cells in response to cell activation largely originates from the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase homolog dual oxidase at the apical surface, and has been under-studied.^{2,3} Studies have also shown that extracellular oxidants such as H₂O₂ are capable of directly stimulating production of cytokines such as interleukin 8 (IL-8) in human airway epithelial cells.^{2,4,5} IL-8, a potent chemoattractant for polymorphonuclear leukocytes (PMNs), is found in increased concentrations in the

nasal secretions of human control cohorts with common colds.^{6,7} Furthermore, a direct correlation has been shown between the severity of nasal symptoms and concentration of IL-8 in the nasal secretions.⁶ Bautista et al.⁸ showed that IL-8 directly contributed to mucin overproduction by regulating the mucin 5AC gene (*MUC5AC*) expression at the posttranscriptional level in a human bronchial epithelial cell culture model. Joo et al.⁹ elegantly showed that dual oxidase 2 (DUOX2)-mediated H₂O₂ generation regulates host defense by enhancing production of proinflammatory mediators (production of IL-8 and *MUC5AC*) and stimulating adaptive immune responses when the mucosal barrier is involved by infection.

Approximately 20 million cases of acute rhinosinusitis (ARS) occur each year, and chronic rhinosinusitis (CRS) exhibits a lifetime prevalence of 14% to 16% of the U.S. population.^{10–12} Epidemiologically, an association between poor air quality and increased prevalence of sinusitis has been established.¹³ A recent cross-sectional study using 2 national datasets identified a relationship between increased fine particulate exposure and the development of sinusitis among adults.¹⁴ The relationship between particulate matter (PM) exposure, production of reactive oxygen species (ROS) in the airways, and development of airway inflammatory disease with mucus production has not been comprehensively investigated. We hypothesized that exposure of control nasal tissue to PM may produce oxidative stress in the nasal mucosa via the release of H₂O₂ and the proinflammatory cytokine IL-8, as well as upregulation of *MUC5AC*.

Materials and Methods

Subjects, sample collection, and PM reagent

This study was approved by the Institutional Review Board at Stanford University. We harvested normal nasal septal mucosa for tissue culture intraoperatively from patients undergoing endoscopic transnasal surgery for benign pituitary lesions.

Coarse PM with an aerodynamic diameter of 10 μ m (PM₁₀) was obtained as a gift from the laboratory of Dr. Jerold Last at the University of California, Davis, CA. Coarse PM consisting of well-characterized ambient particulate pollution was collected in the Sacramento, CA, area in 2009 using a high-volume sampler (Model GS2310; Andersen INC, Smyrna, GA) and a 4-stage cascade impactor (Series 230; Andersen, Inc.); aluminum substrates were used on each stage. Samples were removed from the aluminum substrates, sealed in 2-mL Eppendorf tubes, and stored at –80°C. PM was suspended in phosphate buffered saline (PBS) 30 minutes before use in experiments.

Study design

Primary human nasal tissue were cultured as outlined below and cell integrity and recovery were monitored by lactate dehydrogenase (LDH) release into medium. Once LDH re-

lease was stabilized, cultured tissue was exposed to ambient air pollution PM. Ambient airborne pollution PM was used as an inflammation-inducing agent because it is a common environmental stressor to which the upper airway epithelium is exposed. Ten nasal tissue specimens were exposed to PM₁₀ for 24 hours, and 5 nasal tissue specimens not exposed to PM₁₀ were used as experimental controls. Of the 10 tissues exposed to PM₁₀, 5 were incubated with diphenyleneiodonium sulfate (5 μ M DPI; an NADPH oxidase blocker) for 3 hours after 24-hour exposure to PM₁₀. Then, supernatants from each tissue culture well (n = 15) were collected for immediate H₂O₂ assay. For the cytokine assay, another 15 nasal tissue were divided into 3 groups: the first 5 nasal tissue specimens were exposed to PM₁₀ (20 μ g/cm²) for 24 hours, another 5 tissue specimens were exposed to a higher concentration of PM₁₀ (40 μ g/cm²) for 24 hours, and the last 5 tissue specimens were not exposed to PM₁₀ and were used as controls. After 24 hours, supernatant from each well of cultured tissue was collected for cytokine Luminex assay as outlined below in the Luminex multiplex analysis section.

Human nasal tissue culture system

The biopsied tissue was removed of any tissue debris or blood, and divided into 4-mm $\times$ 4-mm $\times$ 2-mm pieces with a 15-blade scalpel under a dissecting scope. The tissue was then placed on a 0.4- μ m polytetrafluoroethylene (PTFE) membrane (Millicell-CM; Millipore, Cork, Ireland) and immersed in 1.7 mL of culture medium consisting of 50% Dulbecco's Modified Eagle Medium High Glucose (Invitrogen, Carlsbad, CA), 25% Hank's solution (Invitrogen), 50 U/mL penicillin G (Invitrogen), and 40 μ g/mL streptomycin (Invitrogen) in a 60-mm $\times$ 15-mm tissue culture dish (Corning, Corning, NY). Each piece of tissue was placed on a single culture dish, and was oriented on the membrane such that the epithelial side was exposed to the air in a humidified incubator at 37°C with 5% CO₂. The culture media was changed daily.

Lactate dehydrogenase assay

To determine optimal recovery of cell integrity after tissue retrieval and culture, culture medium was tested for the presence of released lactate dehydrogenase (LDH). A 96-well LDH Cytotoxicity Assay Kit (Cayman Chemical, Ann Arbor, MI) was used, with the following modifications: on the day of the assay, 50 μ L of culture medium was removed and added to 50 μ L of Substrate Mix. Absorbance at 490 nm was obtained with an Epx Precision Microplate Reader (Molecular Devices, Sunnyvale, CA). We measured the baseline LDH levels 24 hours after tissue explant; these were again repeated each subsequent 24-hour period for 5 days to determine cell recovery and to identify the optimal window in which to perform experiments.

Luminex multiplex analysis

Human 51-plex kits were purchased from Affymetrix (Santa Clara, CA) and used according to the manufacturer's recommendations with modifications in the next sentence. Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 hours followed by overnight incubation at 4°C. Plates were vacuum-filtered and washed twice with buffered wash solution, followed by incubation with biotinylated detection antibody for 2 hours at room temperature. Samples were then filtered and washed twice earlier in this paragraph and resuspended in streptavidin-phycoerythrin (PE). After incubation for 40 minutes at room temperature, 2 additional vacuum washes were performed, and the samples were resuspended in reading buffer. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower limit of 100 beads per sample per cytokine. Data were analyzed using MasterPlex software (Hitachi Software Engineering America Ltd., MiraiBio Group, South San Francisco, CA), and are reported as mean fluorescence intensities (MFIs).

Ex vivo measurement of H₂O₂

H₂O₂ production was measured using the Amplex Red hydrogen peroxide assay kit (Invitrogen, Eugene, OR). Samples from all groups were collected in an equivalent manner. Supernatants (standard curve samples, control and experimental samples) were mixed with buffer solution of 100 μ M Amplex[®] Red reagent and 0.2 U/mL horseradish peroxidase (HRP) to a final volume of 100 μ L. Amplex Red reacts in a 1:1 stoichiometry with H₂O₂ to produce red fluorescent resorufin, which was detected using a fluorescence plate reader after 30 minutes and calibrated against a standard curve generated from serial dilutions of H₂O₂.

Hematoxylin and eosin and immunofluorescent staining

Hematoxylin and eosin (H&E) and immunofluorescent staining were performed in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, CA). Samples were cut into 9- μ m sections using a cryomicrotome (CM1950; Leica Microsystems Nussloch GmbH, Nussloch, Germany). H&E with Alcian blue staining was performed using standard techniques. Results of H&E and Alcian blue staining of slides were obtained using light microscopy (Olympus DMLS, Japan). For immunofluorescent staining of cytokeratin (Krt) 8, Krt14, and MUC5AC, monoclonal mouse anti-human antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. The sections were washed and blocked in 10% goat serum for 1 hour at room temperature and then incubated with the primary antibody overnight at 4°C. The sections were then washed and incubated for 1 hour at 37°C with a cyanin 5 (CY5)-labeled donkey anti-mouse immunoglobulin G (IgG) antibody (1:100; KPL, Inc., Gaithersburg, MD). 4',6-

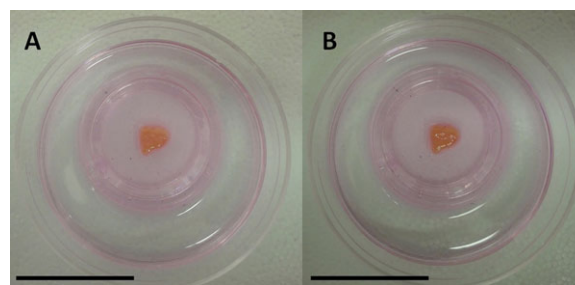


FIGURE 1. Human nasal tissue culture system. Gross appearance of cultured nasal tissue (septum) appeared viable without significant discolorations or tissue shrinkage over 7 days in the culture environment. (A) Day 0. (B) Day 7. Scale bar 3 cm.

Diamidino-2-phenylindole (DAPI) was used as a counterstain to identify all cellular nuclei in the tissue specimen. Immunofluorescence microscopy was performed using confocal fluorescence microscope (Zeiss LSM 710 Spectral Confocal Microscope, Zeiss, Germany).

Statistical analysis

Statistical analysis was accomplished using SPSS software (version 17.0; SPSS Inc., Chicago, IL), and $p < 0.05$ was considered significant. Results are given as mean $\pm$ SE. Between-group differences were assessed for significance by 1-way analysis of variance (ANOVA), Kruskal-Wallis 1-way ANOVA, followed by Dunn's test, Mann-Whitney, or the Pearson's chi-square test, as appropriate.

Results

Patient characteristics

Patients were 43.6 ± 3.4 years old ($n = 7$) and underwent an endoscopic transnasal approach for resection of benign, nonsecretory pituitary lesions. There were 3 male and 4 female control subjects. None of the patients had a history of sinusitis/allergy rhinitis, or findings of mucosal thickening findings in radiographic images. Thirty-five tissue samples from nasal septa were cultured in a single-well system (Fig. 1A). Grossly, the cultured nasal tissue appeared viable without significant discolorations or tissue shrinkage over 7 days in the culture environment (Fig. 1B).

Histologic findings of cultured human nasal tissue

Histological evaluation with H&E staining demonstrated that the overall cellular architecture was generally well-maintained on day 7 of tissue culture. The epithelium remained largely intact and submucosal glands were still present (Fig. 2A, B). We also performed immunostaining with Krt14 and Krt8 to validate the viability of the human nasal tissue cultures on day 7. Krt14 is expressed in submucosal glands (SMGs) and basal cells, and has a role in maintenance of cell proliferation potential in the basal layer of stratified epithelia and SMGs.¹⁵ Krt8 is a marker of cellular differentiation.¹⁶ The sinonasal tissues expressed both of these proteins: Krt14 was more prominently stained in the

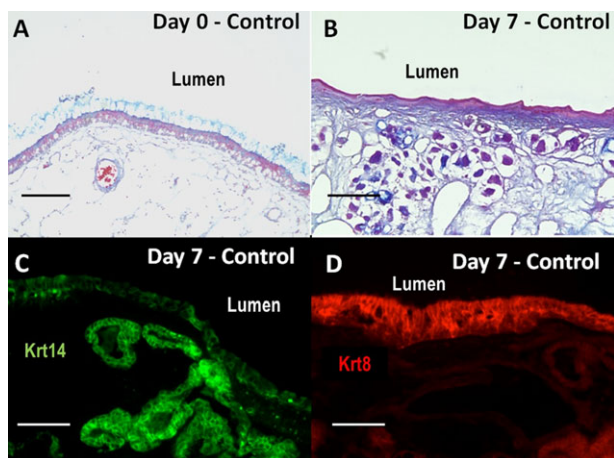


FIGURE 2. Histologic assessment of cultured human nasal tissue. Hematoxylin and eosin staining of nasal tissue on day 0 (A) ($\times 100$, scale bar 100 μm) and day 7 (B) ($\times 100$, scale bar 100 μm). Overall cellular and tissue architecture was generally well-maintained over 7 days in tissue culture. Krt14 (C) and Krt8 (D) were detected in human nasal tissue on day 7 using immunofluorescence microscopy. (C) Krt14 was more prominently detected in the submucosal glands and basal cell layer ($\times 200$, scale bar 50 μm). (D) Krt8 expression was restricted to the luminal layer of stratified epithelium ($\times 200$, scale bar 50 μm). Krt = cytokeratin.

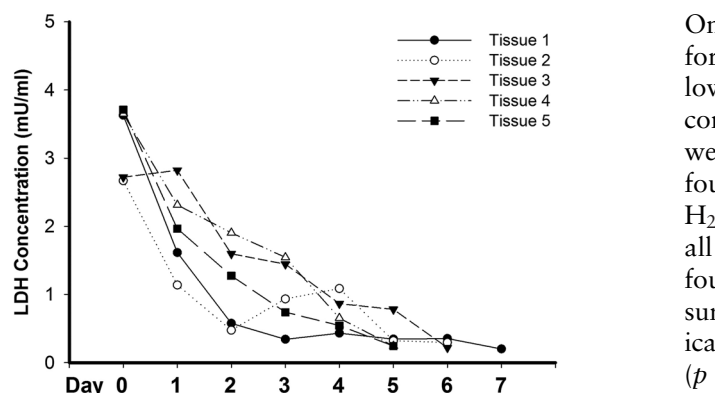


FIGURE 3. Extracellular LDH release by cultured human nasal tissue. LDH was released from nasal tissue over a period of 1 week in culture ($n = 5$) and a rapid reduction in LDH levels was noticed within 2 to 3 days. LDH = lactate dehydrogenase.

SMG and basal cell layer (Fig. 2C) and Krt8 was strongly positive in the pseudostratified surface epithelium (Fig. 2D)

Extracellular LDH release by cultured human nasal tissue

The release of LDH into the extracellular medium is indicative of cell membrane disruption. We measured the levels of LDH in the growth medium of nasal tissue over a period of 1 week in culture condition ($n = 5$) to determine the cellular recovery following surgical resection. The explants showed a rapid reduction in LDH levels within 2 to 3 days in culture, followed by stable LDH levels (Fig. 3). At day 4, LDH had fallen to low levels and tissues were considered felt to be usable for experimentation.

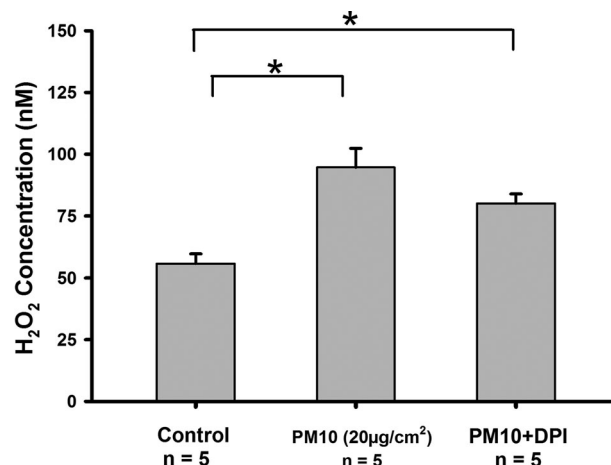


FIGURE 4. Hydrogen peroxide (H_2O_2) release after exposure to PM10. All sinonasal tissue cultures released H_2O_2 into the medium. Exposure to PM ($20 \mu\text{g}/\text{cm}^2$) increased H_2O_2 levels significantly ($94.6 \pm 7.7 \text{ nM}$) compared to untreated controls ($55.8 \pm 4.0 \text{ nM}$; $p = 0.001$). PM-induced H_2O_2 production was partially inhibited by DPI ($80.1 \pm 3.8 \text{ nM}$). $*p < 0.05$. DPI = diphenyleneiodonium sulfate; PM10 = particulate matter with an aerodynamic diameter of 10 μm .

H_2O_2 release after exposure to PM10

On day 4 of culture, 5 nasal tissue were exposed to PM10 for 24 hours, 5 were exposed to PM10 for 24 hours followed by DPI treatment, and 5 were left as untreated controls. Supernatants from each well of cultured tissue were collected; Figure 4 shows the H_2O_2 concentrations found in each group. H_2O_2 was detected in all groups and H_2O_2 concentrations were significantly different between all groups. In supernatants from control tissue ($n = 5$), we found an H_2O_2 concentration of $55.8 \pm 4.0 \text{ nM}$. Exposure to PM10 ($20 \mu\text{g}/\text{cm}^2$) increased H_2O_2 levels significantly ($94.6 \pm 7.7 \text{ nM}$) compared to untreated controls ($p = 0.001$). With an extra 3-hour exposure to DPI after exposure to PM10 ($20 \mu\text{g}/\text{cm}^2$), PM-induced H_2O_2 production was partially inhibited by DPI ($80.1 \pm 3.8 \text{ nM}$, $n = 5$), indicating a cellular NADPH oxidase origin for H_2O_2 .

Upregulation of IL-8 and MUC5AC after exposure to PM10

On culture day 4, nasal tissue samples ($n = 15$) were divided into 3 groups: the first 5 nasal tissue samples were exposed to PM10 ($20 \mu\text{g}/\text{cm}^2$) for 24 hours, another 5 tissue samples were exposed to a higher concentration of PM10 ($40 \mu\text{g}/\text{cm}^2$), and the last 5 tissue samples were not exposed to PM10 and were used as a control. On culture day 5, supernatants from each well of cultured tissue were collected for the cytokine Luminex assay. Figure 5 shows IL-8 concentrations found in supernatants of cultured nasal tissue. Exposure to PM10 increased IL-8 levels in a dose-dependent fashion. In nasal tissue exposed to PM10 ($20 \mu\text{g}/\text{cm}^2$), IL-8 concentrations in the supernatants ($5002 \pm 1327 \text{ MFI}$, $n = 5$) increased more than 2-fold compared to controls ($2301 \pm 412 \text{ MFI}$, $n = 5$). Nasal tissue exposed

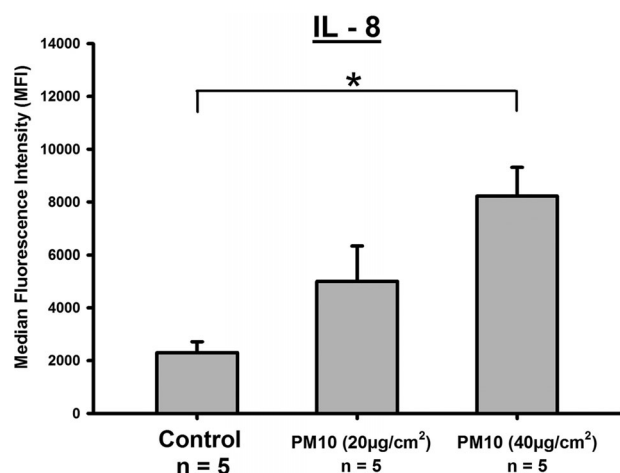


FIGURE 5. Extracellular IL-8 levels in cultured human nasal tissues. Exposure to PM10 increased IL-8 levels in a dose-dependent fashion (control = 2301 ± 412 MFI; $20 \mu\text{g}/\text{cm}^2 = 5002 \pm 1327$ MFI; $40 \mu\text{g}/\text{cm}^2 = 8219 \pm 1090$ MFI; $p = 0.022$). * $p < 0.05$. IL-8 = interleukin 8; MFI = median fluorescence intensity; PM10 = particulate matter with an aerodynamic diameter of $10 \mu\text{m}$.

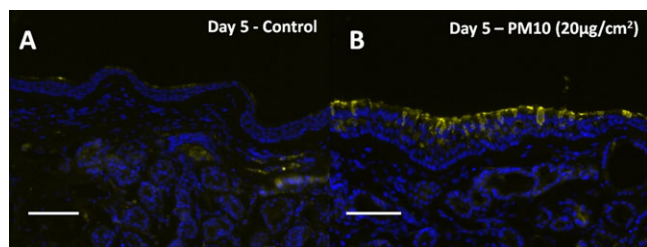


FIGURE 6. (A, B) Localization of MUC5AC immunoreactivity. Markedly higher MUC5AC immunoreactivity was noted in human nasal tissue after exposure to PM10 in immunofluorescent staining. Blue: DAPI; yellow: MUC5AC; magnification $\times 100$; scale bar $100 \mu\text{m}$. DAPI = 4',6-diamidino-2-phenylindole; MUC5AC = mucin 5AC; PM10 = particulate matter with an aerodynamic diameter of $10 \mu\text{m}$.

to a higher concentration of PM10 ($40 \mu\text{g}/\text{cm}^2$) showed IL-8 levels more than 3.5-fold higher (8219 ± 1090 MFI; $n = 5$) than controls ($p = 0.022$). Using immunofluorescent staining, MUC5AC mucin was expressed in surface epithelia of human nasal tissue culture, but was absent in the sub-mucosal glands (Fig. 6). MUC5AC expression was mostly detected in the apical/luminal region and markedly higher MUC5AC immunoreactivity was noted in human nasal tissue after exposure to PM10 compared to controls on day 5.

Discussion

This study demonstrates that PM increases the levels of H_2O_2 and IL-8 in the supernatants released from nasal tissue, indicating both oxidative stress and a proinflammatory state after PM exposure. To our knowledge, this is the first study to show oxidative stress in human nasal tissue after PM exposure.

Dockery and Pope¹⁷ showed a strong correlation between the levels of ambient air particles and mortality; total mortality increased by 1% for each $10 \mu\text{g}/\text{m}^3$ increase in PM10,

respiratory mortality increased by 3.4%, and cardiovascular mortality increased by 1.4%.^{17,18} Exposure to PM is recognized to cause acute respiratory symptoms.^{19,20} The health effects induced by PM result from an inflammatory response caused by the penetration and deposition of particles into the respiratory tract including the sinonasal mucosa.²¹ This response affects normal respiratory function, mucociliary clearance, and other host defense mechanisms, and is a plausible explanation for inflammatory respiratory disorders such as CRS, which was found to be associated significantly with PM10.²¹⁻²³ In this study, PM10 exposure was used as a surrogate noxious stimulus to bad air quality. Based on the study by Wegesser and Last,²⁴ a dose of $50 \mu\text{g}$ is approximately equivalent to the amount of coarse PM that would be inhaled during 1 to 2 week of exposure to PM at the average coarse PM concentration. PM10 exposure in this study appeared to be similar to that of actual air quality.

The 2 large NADPH oxidase (NOX) homologues, dual oxidase 1 (DUOX1) and DUOX2, are highly expressed in the respiratory epithelium.²⁵ Because DUOX is located primarily at the apical surface of polarized airway epithelia where it mediates luminal production of H_2O_2 , it likely functions as a component of innate host defense by producing H_2O_2 as a substrate for locally secreted lactoperoxidase (LPO) to generate antimicrobial oxidants.^{3,26,27} Joo et al.⁹ demonstrated that DUOX2-mediated H_2O_2 generation regulates host defense by enhancing production of proinflammatory mediators (production of IL-8 and MUC5AC) and stimulates adaptive immune responses when the mucosal barrier is irritated by infection. Our previous study using human sinusitis tissue also showed significant upregulation of DUOX expression in CRS, and upregulated levels of DUOX correlated tightly with the H_2O_2 content of nasal secretions.²⁵ The respiratory epithelium is a major source of the chemokine IL-8, which plays a pivotal role in controlling neutrophil and monocyte chemotaxis toward sites of infection, and it may also be involved in epithelial regeneration in response to injury.^{2,28} Epithelial production of IL-8 is enhanced in response to many diverse stimuli, including bacterial and viral triggers that activate Toll-like-receptors (TLRs), various environmental oxidants, and other biological mediators, such as neutrophil elastase or lipid mediators.^{2,29-33} Furthermore, IL-8 is found at increased concentrations in the nasal secretions of control tissues in humans with common colds.^{6,7} Studies have also shown that IL-8 directly contributed to mucin overproduction by regulating MUC5AC expression in a human airway epithelial cell culture model, generating more viscous mucus.^{8,9} A study of ours (Cho, unpublished work) also showed an upregulation of MUC5AC in diseased airway epithelium, with significantly higher IL-8 levels in CRS patients compared to normal controls. Reduced H_2O_2 levels after treatment with the NADPH oxidase inhibitor DPI suggests that H_2O_2 released into the ASL after exposure to PM originated from a NADPH oxidase. The development of selective pharmacological

inhibitors of NADPH oxidases, DUOX1, and DUOX2, or reducing airborne PM may be a potential therapeutic target to control airway inflammation and decrease mucus production.

We feel that the nasal explant system described herein provides a reliable cell based assay system, given the preservation of tissue integrity along with cellular epithelial markers. The ex vivo assay using cultured human nasal tissue described herein offers a simple way to study the local and cellular responses of sinonasal tissues after exposure to PM10. The extracellular level of LDH reliably represented the degree of cell disruption and correlated well with histology.³⁴ Collectively, our results provide evidence that oxidative stress mediated in part by an NADPH-related mechanism may be a factor in the development and progression of PM-induced inflammatory responses, including IL-8 production and MUC5AC expression in the upper airway epithelium.

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Conclusion

Nasal culture explant is a reliable human airway cell model to study the impact of PM on airway health, given preserved tissue integrity and cellular markers. Exposure to PM increased the levels of H₂O₂ released by sinonasal tissue, indicating oxidative stress in part by activating a normal cellular defense mechanism (an NADPH-related pathway). Exposure to PM resulted in elevated IL-8 levels and mucin production in tissue explants. Efforts to reduce airborne PM may lead to reduced H₂O₂ and mucin production in sinonasal epithelium. ☯

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