Effects of Wood Smoke Constituents on Mucin Gene Expression in Mice and Human Airway Epithelial Cells and on Nasal Epithelia of Subjects with a Susceptibility Gene Variant in Tp53

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BACKGROUND: Exposure to wood smoke (WS) increases the risk for chronic bronchitis more than exposure to cigarette smoke (CS), but the underlying mechanisms are unclear.

OBJECTIVE: The effect of WS and CS on mucous cell hyperplasia in mice and in human primary airway epithelial cells (AECs) was compared with replicate the findings in human cohorts. Responsible WS constituents were identified to better delineate the pathway involved, and the role of a tumor protein p53 (Tp53) gene polymorphism was investigated.

METHODS: Mice and primary human AECs were exposed to WS or CS and the signaling receptor and pathway were identified using short hairpin structures, small molecule inhibitors, and Western analyses. Mass spectrometric analysis was used to identify active WS constituents. The role of a gene variant in Tp53 that modifies proline to arginine was examined using nasal brushings from study participants in the Lovelace Smokers Cohort, primary human AECs, and mice with a modified Tp53 gene.

RESULTS: WS at 25-fold lower concentration than CS increased mucin expression more efficiently in mice and in human AECs in a p53 pathway-dependent manner. Study participants who were homozygous for p53 arginine compared with the proline variant showed higher mucin 5AC mRNA levels in nasal brushings if they reported WS exposure. The WS constituent, oxalate, increased MUC5AC levels similar to the whole WS extract, especially in primary human AECs homozygous for p53 arginine, and in mice with a modified Tp53 gene.

DISCUSSION: The potency of WS compared with CS in inducing mucin expression may explain the increased risk for chronic bronchitis in participants exposed to WS. Identification of the responsible compounds could help estimate the risk of pollutants in causing chronic bronchitis in susceptible individuals and provide strategies to improve management of lung diseases. https://doi.org/10.1289/EHP9446

Introduction

Air pollution is the biggest environmental cause of death worldwide and accounts for ~5–6 million deaths every year (GBD 2013 Risk Factors Collaborators et al. 2015). Chronic lung diseases are primarily caused by chronic exposure to cigarette smoke (CS) but also by biomass smoke in high-income and in low/middle-income countries, respectively (Liu et al. 2016; WHO 2013). However, not only does indoor household pollution but outdoor pollution stemming from forest fires and traffic emissions likewise contribute to respiratory diseases. Forest fires alone can account for up to 25–30% of the emissions of fine particulate matter [PM $\leq$2.5 µm in aerodynamic diameter (PM$_{2.5}$)] (Perrone et al. 2012). The contribution of forest fires to air pollution is predicted to increase given that the frequency and intensity of wildfires is expected to rise owing to climate change (Oliveira et al. 2020; Ramo et al. 2021). Because wood smoke (WS) from forest fires is becoming more frequent during summer months, even in traditionally wet areas, not only households in low-income countries, but also millions of people in high-income countries, are consistently exposed to WS and experience respiratory problems (Kang et al. 2021). In addition, exposure to WS indoors has, on average, ranged from 0.05 to 1 mg/m$^3$ total particulate matter (TPM) concentration (Ward et al. 2017) over long periods, unlike exposure to CS, which is short-term but intense; a study evaluating the side-stream emissions of tar from 15 brands of cigarettes found a range of 15–40 mg per cigarette and 2.5–19 mg/cigarette in the mainstream smoke (Rickert et al. 1984).

Meta-analyses have demonstrated that household air pollution from WS not only increases the risk for chronic obstructive pulmonary disease (COPD) exacerbations (CDC 2010; Mannino and Buist 2007; Vestbo et al. 2013) and pneumonia (Dherani et al. 2008; Salvi and Barnes 2009), but it more than doubles the risk of COPD and chronic bronchitis (Hu et al. 2010; Kurmi et al. 2010; Po et al. 2011), and increases emergency room visits, hospitalizations (Peng et al. 2009), and mortality (Bell et al. 2009; Dominici et al. 2006; Mannino et al. 2002). Risk for chronic bronchitis increased drastically with years of cooking and WS exposure (Pérez-Padilla et al. 1996). Chronic bronchitis is a disease of large airways, defined by a persistent chronic cough and sputum production for at least 3 months per year for 2 consecutive years (GOLD 2001).

Computed tomography scans of female former cigarette smokers with COPD has suggested that the airway-predominant COPD phenotype manifested by air trapping is associated with biomass exposure, whereas the emphysematous COPD phenotype is associated with CS (Camp et al. 2014). In another study, with 27 and 21 individuals, matched by age and place of origin, with unique exposure to biomass smoke and CS, respectively, mild fibrosis in the lung parenchyma and bronchi was more common in women with WS-associated lung diseases, but emphysema and goblet cell metaplasia was more common in cigarette smokers (Rivera et al. 2014).
Persons with a longer duration of chronic bronchitis symptoms are at risk for accelerated decline in lung function (Allinson et al. 2016; Vestbo et al. 1996) and were found to have a higher risk of hospitalization compared with persons with a similar level of airflow limitation who did not have chronic bronchitis (Vestbo et al. 1996). They also had increased exacerbation (Kim et al. 2011) and hospitalization (Vestbo et al. 1996) rates compared with persons with a similar level of airflow limitation who did not have chronic bronchitis. We found that self-reported exposure to WS was associated with a higher risk for chronic bronchitis symptoms and lower lung function in humans (Sood et al. 2010) and that mice exposed to WS had higher levels of smoke-induced inflammation (Awji et al. 2015).

Although all smokers develop an inflammatory response, chronic bronchitis was only observed in a subset of heavy smokers in one study (Lapperre et al. 2004), and persistent chronic bronchitis in former smokers may be due to susceptibility genes and exposure to pollution that do not allow the resolution but, rather, promote persistent inflammation (Lapperre et al. 2004). Despite the importance of chronic bronchitis, a limited number of studies have elucidated the mechanisms of WS-induced mucin production or identified susceptibility genes that enhance the risk for WS-induced chronic bronchitis.

Our previous studies in mice showed that hyperplastic mucous cells were sustained by the anti-apoptotic protein, Bcl-2 (Harris et al. 2005), and that blocking Bcl-2 reduced mucous cell hyperplasia (Chand et al. 2017). A subsequent effort to understand the regulation of Bcl-2 identified that p53 reduced Bcl-2 mRNA half-life and that p53 variants defined by a change in amino acid at codon 72 differentially interact and degrade Bcl-2 mRNA. The p53^Arg variant did not interact and reduce Bcl-2 mRNA stability as efficiently as the p53^Pro variant, but by interacting with the promoter, it enhanced the promoter activity of sterile alpha motif pointed domain containing E-twenty-six transcription factor (SPDEF) more efficiently and ultimately drove mucous cell differentiation (Chand et al. 2014).

We found that WS increased the risk of chronic bronchitis by 56% in the Lovelace Smokers’ Cohort (LSC), a well-characterized cohort of smokers living in New Mexico (Bruse et al. 2011; Hobbs et al. 2017; Lange et al. 2015). Because the cohort we studied were all cigarette smokers, this finding suggested that WS has an effect that is different from that caused by cigarette smoking. This assumption was confirmed by laboratory findings in which mice exposed to 250 mg/m^3 CS for 6 h and to 10 mg/m^3 WS for an additional 2 h showed enhanced inflammatory responses compared with mice exposed to either WS or CS alone (Awji et al. 2015). Because we noticed enhanced inflammation, the present study was initiated to determine whether WS may cause enhanced expression of mucins and affect changes to the airways in a manner that CS does not. Therefore, mice and airway cells from mice and humans were exposed to WS or CS to compare their potency in inducing mucin gene expression. Further, we investigated the pathway by which WS induces mucin gene expression and identified the WS constituents responsible for mucin gene expression. We also used primary cells from mice and humans who have a modified p53^Pro or p53^Arg variant to determine whether this p53 variant differentially drives mucin gene expression in response to WS and its constituents.

**Methods**

**Study Population**

The LSC has study participants who are men and women from the Albuquerque, New Mexico, metropolitan area. Inclusion criteria for entry into the current study were being 40–75 years of age and current or former cigarette smokers (with a minimum of 10 pack-years). We have previously described the general demographic information of the LSC (Hunninghake et al. 2009; Lange et al. 2015; Silverman et al. 2011). Participants in the LSC provided informed consent, and all studies using the LSC were approved by the Western Institutional Review Board. All study participants provided information related to demographics, respiratory diseases, and smoking by self-report via a questionnaire. WS exposure was self-reported in response to the question, “Have you ever been exposed to wood smoke for 12 months or longer” as part of the general health survey. However, no additional details about type, intensity, and duration of WS exposure were obtained. Smoking-related variables included smoking in pack-years and current smoking status at the time of testing. Nasal brushings were collected using a Cyto-Pak Soft Brush from the inferior turbinate within each nostril from participants and stored at −80°C in RNasin until use. From 2,273 study participants in the LSC, nasal brushings were available from 1,988 study participants.

**Study Design to Investigate the Role of the p53 Variant Using Nasal Brushings**

To investigate whether mucin gene expression is affected by WS exposure and p53 variant, we first identified 70 study participants for whom nasal brushings were available and who reported exposure to WS. Of these participants, 29 were homozygous for the p53^Arg variant and 41 had at least one p53^Pro variant. Those 70 individuals were then matched by sex, age, Hispanic ethnicity, smoking history, smoking status, body mass index, chronic bronchitis, and COPD (defined by the ratio of forced expiratory volume in one second and the forced vital capacity being <70%) relative to study participants who did not report WS exposure, of whom 29 were homozygous for the p53^Arg variant and 41 had at least one p53^Pro variant (Table S1). The demographic table for the 140 selected participants by WS exposure and p53 variant is shown in Table S2. After quantitative polymerase chain reaction (qPCR) analyses for mucin 5AC (MUC5AC) and SPDEF mRNAs, 20 samples representing 10 each from WS-exposed and nonexposed group were excluded because the ΔCT values were >35 from a 40-cycle amplification and the samples provided unreliable numbers upon replication.

**Exposure Systems for WS and CS**

Mice were exposed in whole-body exposure chambers to mainstream CS from 1R3 research cigarettes (University of Kentucky Tobacco Research and Development Center, Lexington, Kentucky) as was previously described. Mice were acclimated to CS by exposing to 100 mg/m^3 of TPM (CS-100) during the first week, followed by 250 mg TPM/m^3 for 6 h/d, 5 d/wk. For generating WS, ~3 kg of wood (Pinus edulis, the common pinon) was ignited in a wood burning stove with a propane torch and smoke was diverted to the exposure chambers 30 min after the fire was established. Every 15 min thereafter, 1 kg of wood was added to maintain the continuity of the fire during the 2-h exposure period, as previously described (Tesfaigzi et al. 2002). Smoke from the wood burning stove was directed and diluted to the desired concentration with air in a multitered, whole-body exposure chamber at 10 mg/m^3 TPM (H-1000; Lab Products Inc.). A third chamber was used to expose mice to filtered air (FA) as a control. Sterile prebaked quartz (47-mm Tissuequartz) fiber filters ( Pall Gellman) were used to collect TPM to determine the exposure concentrations in the exposure chambers and for preparing extracts. The 100 and 250 TPM mg/m^3 corresponds to 1.3 and 3.3 pack/d cigarette smoking (Finch et al. 1998).
Pollution from cooking indoors can reach 10–20 mg/m³ TPM at peak cooking times (Balmes 2010); therefore, 10 mg/m³ of TPM corresponds to a high indoor air pollution in homes using an open fire for cooking.

**Mice**

Male pathogen-free wild-type C57BL/6J mice were purchased from the Jackson Laboratory. Wild-type mice (p53WT) and mice with a modified proline-rich domain (PRD) in which the four prolines were modified to alanine (p53AXAXA) on a 129J background were provided by G.M. Wahl (Salk Institute for Biological Studies, La Jolla, California) (Toledo et al. 2007) and backcrossed for at least 10 generations into the C57BL/6J background. The p53AXAXA mice were genotyped by standard PCR to demonstrate the different products from wild-type mice using primer sequences, as previously described (Chand et al. 2014). All mice were housed in isolated cages under specific pathogen-free conditions and bred at Lovelace Respiratory Research Institute, Albuquerque, NM and Brigham and Women’s Hospital, Boston, MA. For all studies, each mouse was euthanized using 0.02 mL of Euthasol administered by intraperitoneal injection. Purchased male C57BL/6J mice were randomly assigned to six groups with four mice per group and exposed for 4 and 12 wk to FA, to WS at 10 mg/m³ of TPM for 2/d, to CS for 6 h/d at 250 mg/m³ TPM, or to both. Mice were euthanized 1 d after the final exposure. In addition, female p53WT and p53AXAXA mice were randomly assigned to four groups with three mice per group and exposed to FA or WS at 10 mg/m³ for 6 h for 1 d and euthanized the next day. Exposure to oxalate was performed by intranasal instillation, as previously described (Tesfaigzi et al. 2001). For the oxalate exposure study, equal numbers of male and female p53WT and p53AXAXA mice were randomly assigned to four groups with six mice per group. After isoflurane anesthesia, the mice were intranasally instilled with 100 ng oxalate in 50 µL phosphate-buffered saline (PBS) or vehicle only once a day for 2 consecutive days and euthanized on the third day. All experiments were approved by the Institutional Animal Care and Use Committee and were performed at both the Lovelace Respiratory Research Institute and Brigham and Women’s Hospital, facilities approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International.

Right cranial, middle, and caudal lung lobes were removed from the mice, and the cranial lob tissue was homogenized using a Tissue Lyser II (Retsch) and RNA was isolated with Trizol (Invitrogen) and quantitative real-time (qRT)-PCR were performed with a one-step Taqman PCR Kit (Applied Biosystems). Left lungs were inflated and fixed overnight at 25 cm pressure with zinc formalin (Fisher Chemicals) and kept in ethanol before the preparation of 0.3-cm thick slices for embedding into paraffin. From each mouse, 5-µm thick lung tissue sections were prepared using a Leica microtome and stained with Alcian blue, hematoxylin and eosin, or were immunostained as described below using antibodies to Muc5ac and phosphorylated epidermal growth factor receptor (pEGFR), phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2), or gamma histone family member X (γ-H2AX). In general, two tissue sections per mouse were analyzed. Images of lung sections were captured using a NanoZoomer Digital Pathology slide scanner (Hamamatsu) and epithelial and mucus cells were quantified using Visiopharm Integration System software version 2.16.0.0, by a person who was unaware of the treatment groups.

**Reagents**

Reagents used included polyclonal antibodies for pEGFR (Tyr845) and EGFR (Santa Cruz Biotechnology) and pERK1/2 (T202/Y204), ERK1/2, p53, γ-H2AX (S139) (all from Cell Signaling Technology), and horseradish peroxidase (HRP)-conjugated antimouse (Sigma) or antirabbit secondary antibodies (Jackson Laboratory). We used the First-Strand cDNA Synthesis Kit (Applied Biosystems); gene-specific primers for the qPCR of mouse Muc5ac, Muc5b, and SPDEF and human MUC5AC, MUC5B, SPDEF, and solute carrier family 26, member 9 (SLC26A9) (all from Applied Biosystems). Inhibitors used were those related to sodium/glucose cotransporter 1 (Phlorizin), adenosine triphosphate (ATP)-sensitive potassium channels (glyburide), and cystic fibrosis transmembrane conductance regulator [CFT; GlyH-101; an inhibitor of both phospholipase A2 as well as solute carrier family members (SLCs)], niflumic acid [an inhibitor of ERK1/2 (U0126), pEGFR (AG1407), and all sugar compounds] (all from Sigma).

**Cell Culture**

Primary human airway epithelial cells (AECs) from several donors were provided by H.W. Chu (National Jewish Health, Denver, Colorado). Cells were collected from healthy volunteers who needed bronchoscopy to confirm or exclude respiratory diseases and were stored in a deidentified manner in the National Jewish Health database, and all participants provided written informed consent. The use of human AECs was approved by the institutional review boards at National Jewish Health and at Brigham and Women’s Hospital and a consent form is available. These primary cells were maintained in bronchial epithelial growth medium (BEGM) and small AEC growth media (SAGM) (both from Lonza) supplemented with growth factors (BEGM and SAGM Singlequots; Lonza) at 37°C under an atmosphere containing 5% carbon dioxide as described by Fulcher et al. (2005). Cells were seeded on 804G-coated plates and grown to 60–70% confluence and ~5 x 10⁵ cells were transferred onto each 12-well Transwell membrane (Corning) to allow differentiation in an air–liquid interface condition. Once cells reached complete confluence, over 1–2 d, the medium from the top compartment was removed and cells were fed only from the bottom compartment to allow the air–liquid interface differentiation to occur over 2–3 wk. Mouse AECs were harvested and cultured as previously described (You et al. 2002) and treated in the same fashion as human AECs. Mouse AECs were isolated from murine tracheas after overnight digestion with pronase and washing in PBS. After 2 wk of differentiation on the air–liquid interface, cultured human and mouse AECs that were differentiated on Transwell membranes were treated by adding 100 ng/mL WS or CS extract or 100 nM oxalate into the bottom chamber of the Transwell cultures for 24–48 h. Preparation of WS or CS extracts is described below. For CS exposure and analysis for WS- and CS-induced mRNA expression of MUC5AC and SPDEF of primary AECs, five replicates per group (n = 2) with two experimental repeats (N = 2) were used. For investigating the activation of EGFR, ERK1/2, or p53, three independent experimental repeats (N = 3) were analyzed, and in the corresponding inhibition experiments with AG1478 (10 nM), U0126 (100 nM) or Nutlin-3 (100 nM), respectively, compounds were added to the cultures 30 min before treatment with WS extract. Three independent experimental repeats (N = 3) each with two replicates (n = 2) were used for this study. Differentiated cultures were also fixed after treatment to perform immunostaining for MUC5AC, as described below, in two replicates (n = 2) from four independent experiments (N = 4). Mouse AECs from wild-type and tumor protein p53 (Tdp53) knockout mice treated with WS, three replicates per group (N = 3) with three experimental repeats (N = 3) were used. Human embryonic kidney 293 (HEK293T) cells were purchased from American Type Culture Collection and were
maintained in Dulbecco’s Modified Eagle Medium (Sigma) with 10% fetal bovine serum (Corning) and 1% penicillin-streptomycin (Corning).

**Preparation of WS and CS Extracts**

WS and CS particulate matter were collected onto sterile prebaked quartz (47-mm Tissuequartz) fiber filters ( Pall Gellman), with an average weight of 1–2 mg TPM/filter, and were stored at −80°C in a petri dish rapped with aluminum foil. The filters were incubated in BEGM for 60 min at 37°C to prepare a concentration of 20–40 µg/mL. The stock solution was sterilized by filtering through 0.2-µm pore sized filters (Millipore) and diluted to concentrations ranging from 10 ng/mL to 10 µg/mL for treating cells.

**High-Performance Liquid Chromatography Fractionation of WS Extract and Mass Spectrometry**

For WS fractionation preparation with high-performance liquid chromatography (HPLC), a working solution of 100 µL from a 100-µg/mL concentration was prepared by extracting the filter with WS TPM with dimethyl sulfoxide (DMSO) and fractionated using an Agilent model 1100 HPLC system with a Rheodyne 7725i manual injector and ChemStation software (version A.09.01 or 09.03; Agilent Technologies). For the analyses, a Luna 5-µm C-18 column (4.60 mm ID × 150 mm; Phenomenex Inc.) was used. The mobile phase consisted of purified water with 0.025% formic acid, acetonitrile with 0.025% formic acid, and 10 mM ammonium acetate in a gradient elution with a total run time of 10 min per injection. Every minute, 1-mL fractions were collected, and after brief evaporation of the mobile phase, were kept at −80°C in a petri dish rapped with aluminum foil. The filters stock solution was sterilized by filtering through 0.2-µm pore sized filters (Millipore) and diluted to concentrations ranging from 10 ng/mL to 10 µg/mL for treating cells.

For the nonpolar fractions, a 1-mL aliquot was placed in a 4-mL vial and an equal amount of methylene chloride was added. Samples were spiked with a set of internal standards (AccuStandard Inc.) and shaken well for 10 min. The samples were then left for a few hours to separate the fractions. The methylene chloride layer was carefully removed to a separate vial and a small amount of sodium sulfate was added to remove any residual water. Extracts were then filtered again with a 0.2-µm PTFE Whatman filter. Approximately 500 µL of tolune was added and each extract was concentrated using Pierce Reacti-Therm under a gentle flow of ultra-high purity nitrogen to a final volume of 250 µL.

**qRT-PCR**

Total RNA from cultured cells and mouse lung tissues was extracted using the TRIZOL (TRI Reagent; Invitrogen) and concentrations were determined using a NanoDrop 1000 (Thermo Fisher Scientific). RNAsin was first removed from nasal brushings by centrifugation at 200 × g for 10 min, and RNA was isolated using the RNeasy Micro Kit (Qiagen). Target mRNAs were amplified by qRT-PCR in 20-µL reactions on the real-time ABI Prism PCR system (PRISM 7900HT; Applied Biosystems) using the One-Step RT-PCR Master Mix (TaqMan; Applied Biosystems). Relative quantities from duplicate amplifications were calculated by normalizing average threshold cycle (CT) values to the housekeeping gene (CDKN1B) or 18s RNA to obtain the ΔCT of the target gene of interest, and the relative fold change was used for determining the fold differences by subtraction from the control CT value to obtain 2ΔΔCT, as described previously (Schwalm et al. 2008; Mebratu et al. 2008). The sequences of the primers (Applied Biosystems) used are provided in Table S3. In general, 100-ng total RNA was used for the qTR-PCR using a one-step qPCR program (48°C for 15 min, 96°C for 10 min, and 60°C and 96°C for 40 cycles using QuantStudio 3 (ABI).

**SPDEF Promotor Luciferase Activity and Inhibition Assay**

HEK293T cells were transfected with a pGL3 plasmid construct that was generated for a previous study, containing the SPDEF promotor driving a luciferase reporter gene (Chand et al. 2014). The luciferase activity was measured by activating the SPDEF promotor with total WS extract, the HPLC fractions were prepared from the WS extract, and the identified active compounds. Briefly, 50,000 cells per well were seeded in a 12-well dish and 24 h later transfected with the SPDEF promotor luciferase plasmid using Mirus 2020 transfection reagent. Cells were treated with 100 ng/mL WS extract or HPLC fractions of WS extract that were stored at −80°C, or with the identified active compounds individually or in double or triple combinations at 100 nM each, and 18 h after treatment washed once with PBS and lysed by rocking for 15 min with passive lyses buffer (Promega). Cell lysates (20 µL) were transferred in 96-well plates and SPDEF Promoter luciferase activity measured 1 min after adding 100 µL/well of Luciferase Assay Reagent II and adding Stop & Glo Reagent using Fluoroskanto Ascent (Thermo Fisher Scientific) with SpectraMax iD5 Multi-Mode Microplate Reader ( Molecular Devices).

For identifying the involved receptor(s), cells were treated with inhibitors of the sodium/glucose cotransporter 1 (Phlorizin; at 1 and 10 mM), the ATP-sensitive potassium channels (glyburide, at 1 and 100 µM), the cystic fibrosis transmembrane conductance regulator (GlyH-101, at 10 and 50 µM), or inhibitors of both phospholipase A2 and 16 of SLCs (Niiflumic acid, at 1, 10, and 30 µM). These inhibitors were added 30 min prior to activating the SPDEF promotor by the WS extract or the identified WS constituents.
Retroviral Silencing Using Short Hairpin RNA

Retroviral silencing vector encoding for SLC26A9 short hairpin RNA (shRNA) and control (Ctrl) vector were used. SLC26A9-specific shRNA plasmids and control plasmids (Origene Technologies; Cat. nos. TR301620 and TR300024) were directly used in transduced HEK293T cells using Muris 2020 transfection reagent and packaged into retroviral particles using Phoenix cells (ATCC) as specified by the manufacturer’s instructions. After transfecting Phoenix cells with the retroviral vector, the retrovirus-containing supernatant was collected at 48 and 72 h. The filtered (0.45 μm) supernatant that contains the packaged virus was harvested and used to infect human AECs. To generate stable human AECs expressing the SLC26A9 or Ctrl shRNAs, the virus-containing medium was removed from the AECs after 48 h and transduced cells were selected with puromycin 1 μg/mL for another 48 h in normal growth media. The knockdown efficiency of the shRNA in human AECs was confirmed by qPCR, as detailed in the “qPCR” section and shown in Figure S3, using Taqman primers (Applied Biosystems), and reported in Table S3.

Western Blot Analysis

Total protein lysates and cell extracts were prepared and proteins were analyzed by Western blotting, as described previously (Tesfaigzi et al. 1994). Briefly, cytosolic and nuclear fractions were prepared by lysing cells in NP-40 to obtain the cytosolic fraction and extracting the nuclear proteins with a hypertonic extraction buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.8, 50 mM potassium chloride, and 300 mM sodium chloride) in the presence of protease and phosphatase inhibitors, as described previously (Stout et al. 2007). Concentration of protein was determined using the bicinchoninic acid kit (Pierce/Thermo Fisher Scientific) and equal amounts of 40–60 μg of protein extracts were subjected to polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, and detection of protein by chemiluminescence was as described previously (Tesfaigzi et al. 1994). Antibodies and dilutions used for immunoblotting analyses were pEGFR (sc-2,342, 1:500), EGFR (sc-03, 1:1000; Santa Cruz), pERK1/2 (4695S, 1:1000; Cell Signaling), ERK1/2 (9101S, 1:1000), EGFR (sc-03, 1:1000; Santa Cruz), pERK (4695S, 1:100; Cell Signaling), γ-H2AX (2577L, 1:100; Cell Signaling), and MUC5AC (sc-21701, 1:100; Santa Cruz) in blocking solution overnight at 4°C, sections were washed with blocking solution and counterstained using Fluoromount-G (Southern Biotech) for nuclear staining, the fluorescently labeled cells were analyzed using an imaging system (Axioplan 2; Carl Zeiss) equipped with a charge-coupled device camera (ORCA-ER; Hamamatsu Photonics) coupled with a wave-length switch (Lambda DG-4; Sutter Instrument) and acquisition software (SlideBook 5; Intelligent Imaging Innovations).

Statistical Analysis

Grouped results from at least three different experiments were expressed as mean ± standard error of the mean (SEM). All points from the biological and the experiments replicates were presented in the scatter plot. Differences among groups were examined by analysis of variance and t-tests using Prism statistical analysis software (GraphPad Software) and by application of Tukey’s test for multiple comparisons. Differences were considered significant at p < 0.05.

Results

Exposure of Mice and Primary Airway Cells to WS and CS

Mice were exposed for 4 and 12 wk to either CS, WS, or FA as control. Compared with mice exposed to FA, mice exposed to 10 mg/m³ WSY but not to 250 mg/m³ CS had significantly more mucous cells per millimeter basal lamina in the lung tissues (Figure 1A). Immunostaining for Muc5ac protein confirmed that WS- compared with CS-exposed mice displayed higher numbers of mucous cells at both 4 and 12 wk of exposure (Figure 1B). In this study, mice exposed to CS did not have significantly higher Muc5ac or SPDEF mRNA levels compared with FA controls; however, mice exposed to WS showed higher both Muc5ac and SPDEF mRNA levels at 4 and 12 wk of exposure (Figure 1C).

Further, primary human AECs that were differentiated on air–liquid interface cultures were treated for 48 h to 100 ng/mL WS or CS extract. Similar to what was observed in mice, WS but not CS extract-induced expression of both MUC5AC and SPDEF in differentiated human AECs (Figure 1D). The increase of the mucin-related mRNAs was accompanied by higher MUC5AC-protein levels in WS extract-treated cultures (Figure 1E).

Because there are no inflammatory cells present in the differentiated airway epithelial cultures, these findings suggest that WS directly affects the airway epithelium. Several studies have reported that EGFR phosphorylation induces MUC5AC expression (Casalino-Matsuda et al. 2006; Shao et al. 2004), but the role of WS exposure in phosphorylating EGFR in increasing MUC5AC

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gene expression has not been reported. Differentiated primary human AECs were treated with 100 ng/mL WS or CS extract and harvested at 0, 1, 3, 8, 24, and 48 h posttreatment. The CS extract had minimal effect on EGFR phosphorylation at any time point (Figure 2A), whereas cells exposed to the WS extract had significantly higher EGFR levels from 3–48 h (Excel Table S2); higher levels of pEGFR at 1, 3, and 8 h; and higher levels of pERK1/2 starting at 1 h, with more than 3-fold higher levels at 3, 8, 24, and 48 h, although these latter time points were not statistically significantly different from control (Figure 2A; Excel Table S2). In addition, immunostaining of lung tissue sections from mice exposed for 12 wk showed that cells exposed to WS exhibited higher levels of pEGFR and pERK1/2 (Figure 2B). Differentiated human AECs treated with the EGFR or ERK1/2 inhibitors, AG1478 or U0126, respectively showed similar levels of MUC5AC or SPDEF treated with the EGFR or ERK1/2 inhibitors, AG1478 or U0126, p53 activation (Liu et al. 2005), we investigated whether the low concentration of WS extract may affect p53 and the DNA damage repair mechanism. Cells exposed to CS extract expressed higher protein levels of p53 at 1 h that were sustained over 48 h, whereas those exposed to WS extract demonstrated higher p53 levels beginning at 3 h (Figure 2D). CS-exposed cells sustained higher γ-H2AX levels only at 3-h posttreatment, whereas WS-exposed cells exhibited significantly higher levels at 3–8 h (Figure 2D; Excel Table S2) and immunostaining of lung tissue sections from mice exposed for 12 wk showed a lower percentage of γ-H2AX+ cells in WS-exposed vs. CS-exposed mice (Figure 2E). When treated with Nutlin-3, a small molecule that disrupts the p53–MDM2 interaction and stabilizes p53 (Vassilev et al. 2004), WS-induced MUC5AC mRNA levels were augmented in differentiated human AECs (Figure 2F). Further, primary mouse AECs from Tp53−/− mice treated with WS extract had significantly lower Muc5ac mRNA levels than those from Tp53+/− mice (Figure 2G). These findings suggest that the p53 signaling pathway is central in WS extract-induced SPDEF and MUC5AC expression.

**Fractionation of WS to Identify the Constituents Responsible for Mucin Gene Expression Using a Promoter–Luciferase Construct**

A rapid assay was developed for identifying WS components that are responsible for inducing mucous cell differentiation by transfecting HEK293T cells with a SPDEF promoter construct driving a luciferase construct. Cells treated with WS extract at 10 and 100 ng/mL TPM concentrations compared with extracts from filters without WS showed higher SPDEF promoter activity by 2- to 3-fold (Figure S1A). The 1-mL fractions obtained from HPLC were tested at various dilutions in activating the SPDEF promoter. From two independent fractionation experiments, from among 20 fractions, the hydrophilic fractions 1, 2, and 3 showed a 6- to 8-fold higher SPDEF promoter activity compared with extracts from clean filters (Figure 3A). Fractions 5 and 6 showed a 5-fold higher activity, and fractions 8, 9, 10, 12, and 13 showed activities ranging from 2- to 4-fold higher, whereas no difference in SPDEF promoter activity was observed with fractions 14, 15, although significantly different from untreated, was similar in activity as in fraction 14. Fraction 20 was similar to the untreated filter sample (Figure S1B). These results suggested that in fractions 1–3 inducing compounds may have been enriched or inhibitors depleted and that the exact opposite may have happened in fractions 15 or 20. Detailed analyses by GC-MS of
fractions F2 and F15 using internal standards that comprise ∼300 compounds identified hydrophilic and hydrophobic compounds, respectively. The main constituents of the F2 fraction were sugars (Figure 3B), and comparison of each constituent in fractions F2 and F15 with those in the whole WS extract showed that oxalate, levunilate, xylitol, α-D-glucose, mannose, and mannitol were represented at higher levels in the F2 fraction. The concentrations of the identified sugars and their percentages in F2 and F15 fractions are listed in Excel Table S3, and the concentrations per milligram TPM in Table S4. Of the pure sugars, only cells treated with levunilate, oxalate, and xylitol had consistently higher SPDEF promoter activity at concentrations that were found in the WS extract (Figure 3C). We tested the effect of these sugars in combinations of two and found that only levunilate enhanced the effect of oxalate and xylitol, but not of other sugars (Figure 3D). Cells exposed to triple combinations, containing oxalate, glucose, mannose, xylitol, or inositol with any of the other sugars did not significantly differ from the untreated control (Figure 3E). For further studies, we decided to investigate the role of oxalate further given that many reports have identified it in environmental pollutions from forest fires (Jalava et al. 2015; Tomaz et al. 2018). Differentiated human AEC cultures exposed to oxalate had higher MUC5AC and SPDEF mRNA levels (Figure 3F) to extents similar to what was observed by WS extract treatment (Figure 1C), and this observation was also replicated in differentiated mouse AECs (Figure 3G).
Figure 3. Fractionation of wood smoke (WS) extract using high-performance liquid chromatography (HPLC) and identification of WS constituents that induce mucin gene expression using GC-MS. Fractionation of WS and identification of WS. (A) SPDEF promoter construct in PGL3 basic vector transfected into HEK-293 cells 1 d prior to treatment with HPLC fractions of WS or left untreated (NT). SPDEF promoter activity in the cell lysates measured by luminometer. Data from \( n = 2 \) replicates/group in each of \( N = 3 \) experimental repeats. (B) Identified compounds in the active HPLC fractions, F2 and F15, by tandem mass spectrometry. (C) SPDEF promoter luciferase activity after treatment with WS extract or sugars found in fraction 2. Cells were treated with 100 nM of all sugars except for maltitol-β at 10 nM. Data from \( n = 2 \) replicates/group in each of \( N = 3 \) experimental repeats. (D) SPDEF promoter luciferase activity after treatment with WS extract or combinations of sugars found in fraction 2. Data from \( n = 2 \) replicates/group in each of \( N = 3 \) experimental repeats. (E) mRNA expression of MUC5AC or SPDEF from primary human AECs differentiated on Transwell membranes and treated with oxalate 100 nM for 48 h. Data from \( n = 2 \) replicates/group in each of \( N = 3 \) experimental repeats. (F) MUC5AC and SPDEF mRNA levels quantified by qPCR from mouse AECs exposed to 100 nM oxalate or left untreated for 48 h. Data from \( n = 2 \) replicates/group in each of \( N = 3 \) experimental repeats. (G) Human AECs were treated with 100 ng/mL oxalate for indicated times (0, 1, 3, 8, 24, or 48) and p53 and γ-H2AX protein levels measured by Western blotting. Image is representative of \( N = 3 \) experimental repeats. For each figure the mean ± SEM is graphed, difference at **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \) with statistical t-test. For (A–F), luciferase activity and mRNA expression are graphed relative to the untreated control. For (G), protein expression is presented relative to time point zero. Summary data is provided in Excel Table S3. Note: AEC, airway epithelial cell; GC-MS, gas chromatography–mass spectrometry; γ-H2AX, gamma histone family member X; HAEC, human airway epithelial cell; Muc5ac, mucin 5ac; NT, untreated; p53, protein 53; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; SPDEF, sterile alpha motif pointed domain containing E-twenty-six transcription factor.

Identification of the Responsible Cell Surface Proteins for Oxalate-Induced Mucin Expression Using Different Inhibitors

To identify the cell surface receptor responsible for the observed oxalate-induced SPDEF promoter activity and MUC5AC mRNA expression, we used different inhibitors that block sugar and anion transport activity. Cells treated with WS and inhibitors of the sodium/glucose cotransporter 1 (Phlorizin) and the ATP-sensitive potassium channels (glyburide) or cystic fibrosis transmembrane conductance regulator (GlyH-101) did not differ significantly from those with WS alone with regard to SPDEF promoter activity (Figure 4A). However, cells treated with WS and 10 and 30 μM niflumic acid, an inhibitor of both phospholipase A2 and 16 of SLCs, had higher SPDEF promoter activity than those treated with WS extract alone (100 ng/mL) (Figure 4A). SPDEF promoter activity by WS extract or oxalate was higher in AECs treated with niflumic acid in a dose-dependent manner at 1, 10, and 30 μM concentrations (Figure 4B). Among the SLC family inhibited by niflumic acid, only SLC26A9 and SLC26A4 are known to be expressed in AECs (Lohi et al. 2002; Simão et al. 2013), and SLC26A9 is a constitutively active CFTR-regulated channel in bronchial cells (Chang et al. 2009). Therefore, we suppressed SLC26A9 mRNA levels using a short hairpin construct (Figure S3A) and found that SPDEF promoter activity was significantly higher when cells were treated with WS extract or oxalate, compared with short hairpin Control (shCTRL) constructs (Figure 4C). The role of SLC26A9 was confirmed as suppressing SLC26A9 levels using shSLC26A9 in differentiated primary human AECs (Figure S3B) enhanced both WS extract- or oxalate-induced MUC5AC and SPDEF mRNA levels compared with shCTRL-infected cultures (Figure 4D).

Testing of WS-Induced Mucous Cell Metaplasia and Mucin Gene Expression in Humans and Mice and AECs with Modifications in the PRD of p53

We have previously reported that the single nucleotide polymorphism, rs1042522, which substitutes a proline (CC to arginine (CG)) at codon 72 within the PRD of p53, affects Bcl-2 mRNA...
half-life and SPDEF promoter activity (Chand et al. 2014). We evaluated the mRNA levels of MUC5AC in nasal brushings from cigarette smokers who self-reported WS exposure by p53 variant (p53Arg and p53Pro). Although MUC5AC mRNA levels in nasal brushings of WS-exposed combined with nonexposed cigarette smokers were not statistically significant by p53 variant (Figure S4), the ratios of MUC5AC in WS-exposed to nonexposed participants was higher for the homozygous for the p53Arg variant compared with the p53Pro variants combined (Figure 5A). Further, differentiated human AECs homozygous for the p53Arg variant showed higher expression of MUC5AC, but not SPDEF, mRNA when treated with 100 ng/mL WS extract (Figure 5B) and 10 ng/mL oxalate (Figure 5C). Differentiated primary AECs from p53<sup>Pro</sup> mice treated with oxalate had higher expression of Muc5ac but not SPDEF mRNA compared with those from p53<sup>WT</sup> mice (Figure 5D). In addition, p53<sup>Pro</sup> compared with p53<sup>WT</sup> mice exposed to WS for 48 h showed higher levels of Muc5ac and SPDEF mRNAs in their lungs (Figure 5E). Discussion

The present studies demonstrate that treatment of AECs with WS compared with CS exposure results in p53 activation as suggested by higher levels of p53 protein and higher phosphorylation levels of EGFR and ERK1/2. We found that MUC5AC gene expression was driven by the p53 pathway. Further, we showed that a major component of the WS mediating these outcomes was sugars. We propose that this represents a new pathway for mucin gene expression and have evaluated a role for a p53<sup>Arg</sup> variant in this pathway. The observation that WS and its constituents caused higher mucin gene expression in differentiated air–liquid interface cultures supports the idea that these sugars can activate mucin expression pathway independent of cellular infiltrates.

The gases and the TPM generated by cigarettes and wood burning are similar (Pryor 1992; Tesfaigzi et al. 2002), consisting of primarily carbon black particles and organic compounds, many of which have yet to be identified. However, differences in the effects of WS and CS on cells have been reported. Chronic exposure to biomass smoke compared with CS was associated with higher levels of distinct circulating inflammatory cytokine alterations in healthy women (Falñán-Valencia et al. 2020). In addition, extracts of ambient particulate matter from WS compared with CS had a 30-fold greater potency in a mouse skin tumor induction assay (Cupitt et al. 1994; Naheer et al. 2007). These findings suggest that WS could contribute to the pathological mucus overproduction observed in patients with COPD. Consistent with our findings, a computed tomography scan study of participants with COPD suggested that WS exposure and cigarette smoking are associated with the airway and the emphysema-predominant COPD phenotype, respectively (Camp et al. 2014; Ramírez-Venegas et al. 2006). Collectively, our findings establish a mechanistic link to previous reports that exposure to WS causes airway-predominant rather than emphysema-predominant COPD.
Although both WS and CS activated the EGFR/ERK pathway, activation occurred earlier by CS than WS exposure and the stronger activation of the EGFR/ERK1/2 pathway by WS may have roles other than mucin gene expression and needs further investigation. It is well established that mucin gene expression is driven by the EGFR and ERK1/2 activation pathway in response to many stimuli, including CS (Gensch et al. 2004; Lemjabbar and Basbaum 2002), nitric oxide (Song et al. 2007), and matrix metalloproteinases (Deshmukh et al. 2005, 2009). Another study reported that the particulate matter of WS when administered to human bronchiol epithelial cells, activated EGFR and led to MUC5AC expression (Memon et al. 2020). The difference between our findings and that report may be in the components used for activating EGFR. EGFR can be activated at various sites of phosphorylation, leading to different downstream effects (Tong et al. 2014). Because our study focused primarily on water-soluble extracts of WS particulate matter, we found that the sugars modulated p53 rather than the EGFR/ERK1/2 pathway to induce SPDEF and MUC5AC expression. Following double-stranded DNA strand breaks, p53 levels increase because of stabilization (Kubbutat et al. 1997) and histone H2AX is rapidly phosphorylated by kinases at or near the vicinity of the DNA damage sites to form γ-H2AX (Fernandez-Capetillo et al. 2004). Although both WS and CS activated p53, the activation of p53 by CS occurred earlier and the activation by WS lasted over a longer period. This prolonged activation may play a role in driving SPDEF expression and the mucous cell differentiation process.

Using chromosome immunoprecipitation, we previously established that the p53 arginine rather than the p53 proline variant interacted and drove the SPDEF promoter both in human and primary mouse AECs (Chand et al. 2014) and that WS activated the p53 arginine variant. The SPDEF promoter was driven more by the p53 arginine variant compared with the p53 proline variant and led to increased mucin production in human AECs (Chand et al. 2014). The solute carrier 26 gene family located at the plasma membrane transports a variety of monovalent and divalent anions, including oxalate (El Khouri and Touré 2014). The suppression of SLC26A9 in HAECArg cultures led to an increase of WS- and oxalate-induced MUC5AC levels. Although IL-13-stimulated goblet cell metaplasia was similar in both SLC26A9+/+ and SLC26A9−/− mice, SLC26A9−/− mice displayed increased airway mucus volume density and airway mucus obstruction of airways likely because chloride secretion facilitated the mucus obstruction (Anagnostopoulou et al. 2012). Mice were instilled with 5 μg IL-13 on 3 consecutive days, and IL-13 may induce mucin gene expression by mechanisms different from what is observed by oxalate. When the anion exchanger is removed, it is likely that intracellular oxalate levels are increased, thereby increasing the formation of intracellular reactive oxygen species. Because we would expect that oxalate levels would increase in the extracellular space of WS-exposed cells, reducing the levels of SLC26A9 would likely increase intracellular oxalate levels and thereby increase mucin gene expression.

For the chemical analyses, the WS extract was prepared in DMSO to capture not only the hydrophilic but also the hydrophobic compounds. The goal was to test the capacity of all WS components in inducing mucin gene expression. As indicated by the initial studies, the findings confirmed that water-soluble compounds were effective compounds in inducing mucin gene expression. The results support the idea that the effective compounds we had extracted from the filters were unlikely to be in the volatile portion or may have been affected by storage of the

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filters, although future studies are needed to investigate whether other compounds in the volatile portion may also have mucin-inducing properties. Although smoke and filters can be contaminated with lipopolysaccharide (LPS), our studies showed that certain purified sugars activated the SPDEF promoter, leading to higher MUC5AC expression. The concentration of LPS needed to induce MUC5AC expression is in the microgram range (Tokita et al. 2014), whereas the concentrations we are using for oxalate is 100 ng. This discrepancy supports the idea that LPS, as a contaminant in the oxalate preparation, was not causing MUC5AC expression. Oxalate has also been found in the dust from urban air (Jalava et al. 2015), but a more detailed analysis of the proportions in compounds that ultimately lead to mucin production is needed. CS also contains oxalate (Gu et al. 2016); however, whether the proportions of oxalate and inhibitors are present in CS at different proportions and, therefore, inhibit their effect is currently unknown. Further, volatile components, such as acrolein, known to induce mucin expression (Borchers et al. 1999; Deshmukh et al. 2009), were excluded in our study, which focused on extracts from the TPM.

Such analyses will help assess a more detailed documentation of pollutants in epidemiological studies to ultimately determine health effects. Intake of high fructose corn syrup-sweetened soft drinks is associated with prevalent chronic bronchitis in U.S. adults, 20–55 years of age (DeChristopher et al. 2015). The risk for chronic bronchitis and dyspnea in participants with diabetes is increased by 2- to 3-fold, especially in individuals 55–74 years of age (De Santi et al. 2017). However, the mechanisms for circulating sugars increasing the risk for chronic bronchitis may be by increasing intracellular reactive oxygen species through hypoxia-inducing factor–1 (Codo et al. 2020) and different than the mechanism we describe in this study, which involves SLC26A9.

Studies to identify genetic polymorphisms associated with chronic bronchitis have not been reproducible across cohorts (Dijkstra et al. 2015; Lee et al. 2014; Silverman et al. 2002). In addition to the differences in the selection of the comparison groups, the present study suggests that reproducible results may also be hampered by the possibility that, depending on their composition, various pollutants affect different susceptibility genes to increase risk for chronic bronchitis. The present study shows that although WS and CS have largely comparable compositions, their effects on the cell and mucin gene expression are vastly different. Generally, airway pollution measured by respirable particles ≤2.5 nm in aerodynamic diameter was associated with lung function in Chinese adults (Xue et al. 2021). Comparison of participants with only chronic bronchitis to healthy controls by air pollutants is difficult owing to the limited number of participants in even large COPD cohorts. Therefore, detailed and rigorous analyses of pollutants to define environmental exposures, also in occupational settings, and a large number of well-phenotyped individuals are required to elucidate gene–environment interactions.

Our study has several limitations: The direct effect of expressing higher levels of MUC5AC on mucus viscosity has not been studied, and whether WS affects expression of other mucin genes will need to be investigated in the future. Further, this study does not compare whether other wood types and burning conditions have a similar effect as burning pine as described in this study. The effect of increased MUC5AC expression on diseases such as chronic bronchitis needs to be studied in larger well-characterized populations who have documented WS exposure to replicate our epidemiological finding. Studies to replicate our findings using cohorts with reported exposure to dust and fumes, as in the COPDGene Study, showed no association with the Tp53 genotype. However, our findings in mice that modification of the prolines in the PRD of p53 increases mucin production in response to WS and oxalate confirms the finding in humans. This mouse model may provide a tool for more mechanistic investigations into susceptibility to various pollutants and active components. Such controlled studies may provide the opportunity to model polygenic risk interactions and lay the foundation for designing cohort studies with the number of individuals that will allow targeted gene–environment interaction analyses.

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