Online Data Supplement

Use of a Soluble Epoxide Hydrolase Inhibitor in Smoke-induced Chronic Obstructive Pulmonary Disease

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Method:

2.1. Animals

Twelve-week-old male spontaneously hypertensive (SH) rats were purchased from Charles River Laboratories (Portage, MI). After arrival, all animals were housed in polycarbonate cages under a light cycle maintained at a 12-hour light-dark pattern with continuous access to food and water before, during, and after exposures. Animals were acclimated for one week before initialization of TS exposure. Animals were weighed weekly before and after tobacco smoke exposure. All animals were handled according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health, and all procedures were performed under the supervision of the University Animal Care and Use Committee, IACUC approved protocol number 07-12922 (University of California, Davis).

2.2. Tobacco smoke exposure

Groups of four to eight SH rats were exposed to filtered air or to tobacco smoke, at a concentration of approximately 80–90 mg/ m3 total suspended particulates (TSP) for 4 weeks. Whole body exposure to cigarette smoke was performed (6 h/day, 3 days/week) using a TE10 smoke exposure system [E1] that combusts 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, KY) with a 35 ml puff volume of 2 seconds duration, once each minute (Federal Trade Commission smoking standard).

2.3. Drugs and Delivery

The soluble epoxide hydrolase inhibitor (t-TUCB):*trans*-4-{4-[3-(4-**t**rifluoromethoxy-phenyl)-ureido]-**c**yclohexyloxy}-benzoic acid and phosphodiesterase type 4 inhibitor Rolipram: (*R/S*)-4-(3-cyclopentyloxy-4-methoxy-phenyl)pyrrolidin-2-one were delivered in drinking water one week prior to smoke exposure and continued throughout the 4-week study. The dose used for t-TUCB was 1.5 mg/kg and 0.3mg/kg for rolipram dissolved in PEG 400 and added to drinking water to give a final PEG concentration of 2% (v/v). Age-matched non-smoke-exposed and vehicle-administered animals were used as normal group animals.

2.4. Pulmonary Function Measurements

Eighteen hours post 4 weeks of TS exposure, rats were deeply anesthetized with Ketamine and Xylazine. A midline incision was made over the cervical trachea. Once the rats were tracheo-cannulated, the cannula was then connected to the Scireq Flexivent (Montreal, Canada) positive pressure ventilator, pulmonary mechanics measurement and data acquisition system. Animals were paralyzed by succinylcholine. The lung volume and pressure were measured twice while a standard respiratory cycle was simulated—once with the catheter open to room air, and once with it closed. The regular ventilation was delivered at a frequency of 90 breaths/min with a tidal volume of 10 ml/kg. Lung mechanics was evaluated using a forced oscillation technique. Measures of respiratory system input impedance was obtained that allows for the unique distinction between central and peripheral lung mechanics. A "snapshot

perturbation" maneuver was imposed to measure resistance (R), compliance (C), and elastance (E) of the whole respiratory system (airways, lung, and chest wall). Forced oscillation perturbation was consequently applied, and resulted in Rn (central airway resistance), tissue damping (resistance) (G) and tissue elastance (H).

2.5. Tissue preparation

SH rats were anesthetized with an overdose of sodium pentobarbital following the pulmonary function test. The trachea was cannulated, the left lung bronchus tied, and the right lung lavaged with Ca^{2+}/Mg^{2+}-free Hank's buffered salt solution (HBSS) bronchoalveolar lavage (BAL) fluid (BALF) was collected in tubes and kept on ice prior to further processing. The lavaged lobes from the right lung were frozen in liquid nitrogen and stored at -80°C until use. For histology, the suture on the left lung bronchus was released, and the lung was inflated with 4% paraformaldehyde at 30 cm water pressure for 1 h, which was then followed by an immersion fixation, paraffin embedding and histopathology examination. Five-μm-thick paraffin-embedded tissue sections were deparaffinized and stained with hematoxylin and eosin (H&E) (American Master Tech Scientific, Lodi, CA), and periodic acid–Schiff staining (AB/PAS).

2.6. Bronchoalveolar Lavage (BAL) analysis

The BALF was centrifuged at 250g for 10 min at 4^{o}C and the cell pellet was resuspended in Ca^{2+}/Mg^{2+}-free HBSS [E2]. Total cell number was determined using a

hemocytometer. Cytospin slides (Shandon, Pittsburgh, PA) were prepared using aliquots of cell suspension that were then stained with Hema 3 (Fisher Scientific, Pittsburgh, PA). Cell differentials in BALF were assessed by counting mononuclear cells, neutrophils and eosinophils on cytocentrifuge slides using light microscopy (300 cells counted per sample).

2.7. Cytokine Analysis of lung homogenates

Lung homogenates from rats were analyzed for 24 different cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, IL-18, Eotaxin, G-CSF, GM-CSF, GROKC, IFN-γ, M-CSF, MIP-1α, MIP-3α, RANTES, TNF-α, vascular endothelial growth factor (VEGF), EPO) utilizing a fluorescent bead multiplex assay from Bio-Rad Laboratories (Hercules,CA). The assay was performed according to the manufacturer's instructions. The assay allows for the detection and quantitation of multiple cytokines at the protein level in a single sample using a 96-well microplate format. The standard curve for each cytokine was produced with a sensitivity of less than 10 pg/ml for each cytokine or chemokine.

2.8. Oxylipin measurements by liquid chromatography/mass spectrometry–mass spectrometry

Oxylipins from lung tissues were extracted by solid phase extraction (SPE) on 60 mg Waters Oasis-HLB cartridges (Milford, MA) according to the previously described method [E3]. Then the elutions from SPE cartridges were evaporated using a

Speedvac® system and reconstituted with 50 uL 200 nM 1-cyclohexyl ureido, 3-dodecanoic acid (CUDA) methanol solution. CUDA was used as an internal standard. The liquid chromatography system used for analysis was an Agilent 1200 SL liquid chromatograph series (Agilent Corporation, Palo Alto, CA). The autosamplar was kept at 4 °C. Liquid chromatography was performed on an Eclipse Plus C18 2.1 × 150 mm, 1.8 μm column (Agilent Corporation, Palo Alto, CA). Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile/methanol (84:16) with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 250 μL/min. Chromatography was optimized to separate all analytes in 21.5 min. Analytes were eluted according to their polarity with the most polar analytes, prostaglandins and leukotrienes eluting first, followed by the hydroxy and epoxy fatty acids. The column was connected to a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, Foster City, CA) equipped with an electrospray source (Turbo V). The instrument was operated in negative multiple reaction monitor (MRM) mode. The optimized conditions and the MRM transitions were reported previously [E3]. Quality control samples were analyzed at a minimum frequency of 10 hours to ensure stability of the analytical calibration throughout the analysis. Analyst software 1.5 was used to quantify the results according to the standard curves.

2.9. Histopathology of the lung

Histology was performed using cross-sectional lung tissue slices containing the first and second intrapulmonary airway generations from rats. Five micron thick sections

were cut from paraffin-embedded tissue blocks on a microtome. Sections were placed on glass slides and baked overnight at 37^{o}C. Sections were subsequently deparaffinized and stained with the following: 1) hematoxylin and eosin (H&E) (American Master Tech Scientific, Lodi, CA) for measurement of airspace size of alveolar tissue, and 2) alcian blue/periodic acid-Schiff (AB/PAS) (American Master Tech Scientific, Lodi, CA) for mucous glycoconjugate detection. For each staining assay, a minimum of 6 fields were sampled from the central airway with an Olympus BH-2 microscope in a uniform random manner. To assess the fraction of the epithelial AB/PAS staining and mean linear intercept (MLI), images were projected onto a monitor and overlaid with a test grid generated by stereology toolbox software (Morphometrix, Davis, CA). Volume fraction was calculated as the number of total points hitting the objectives and total points falling within the reference space. Volume fraction = Pm (points of AB/PAS positive staining)/Pe(points of airway epithelium). MLI was calculated from the number of alveolar tissue intersections encountered in sampling templates. MLI = 2 (total number of sampled fields) (total number of test lines on the sampling template) (length of the test line, corrected for magnification) / (total number of intercepts).

2.10. Statistical Analysis: Data were analyzed and graphed using SAS version 8.2 statistical software (SAS Institute, Cary, NC). Data were expressed as mean ± SEM and generally analyzed using two-way ANOVA, and when significance was achieved, a Bonferroni post hoc test was used. For analysis of the correlation between oxylipins with airway obstructions based on FlexiVent measurements, linear regression will be

used to analyze correlations by Pearson's correlation coefficient. The significance level considered will be $p<0.05$.

Reference:

E1. Teague, S.V., K.E. Pinkerton, M. Goldsmith, A Gebremichael, S. Chang, R.A.Jenkins and J.H. Moneyhun. , Sidestream cigarette smoke generation and exposure system for environmental tobacco smoke studies. Inhalation Toxicology, 1994(6): p. 79-93.

E2. Smith, K.R., et al., Inhibition of tobacco smoke-induced lung inflammation by a catalytic antioxidant. Free Radic Biol Med, 2002. 33(8): p. 1106-14.

E3. Yang, J., et al., Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. Anal Chem, 2009. 81(19): p. 8085-93.