On-line Supplement

Aberrantly activated EGFR contributes to enhanced IL-8 expression in COPD airways epithelial cells via regulation of nuclear FoxO3A

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Methods

Primary cells and bronchial sections. This study was approved by the University of Michigan Investigational Review Board. All bronchial segments were obtained from intact and healthy tissue of healthy non-smokers, smokers without COPD or from COPD subjects during lung transplantation. None of the donors had exacerbation at the time of tissue collection. Five out of 6 met symptomatic criteria for chronic bronchitis, whereas 4 had at least one symptomatic exacerbation in the previous year. Tissues were obtained from either University of Michigan (4 healthy non-smokers, 2 healthy smokers and 3 COPD subjects) or National Disease Research Interchange (Philadelphia, PA)) (tissues from 3 COPD and 2 healthy non-smokers). Airway epithelial cells (AEC) isolated tracheobronchial segments of COPD or normal donors were isolated and cultured at passage one as described earlier [1]. Briefly, airway epithelial cells at passage 1 were cultured in transwells under submerged conditions for a week or until confluent. Then, the cells were lifted to air-liquid interface to promote differentiation into mucociliary phenotype. In some experiments, COPD cells differentiated into mucociliary phenotype were treated with 1 μM erlotinib, 1 μM LY294002 (both from Cayman Chemical, Ann Arbor, MI), or 1 μM quercetin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or DMSO (vehicle control) for 2 weeks. Transwells were shifted to new plate, containing fresh media and incubated for 24 h. Basolateral medium was collected for IL-8 determination and cells were used for protein analysis by Western blotting.

Bronchial tissue from healthy non-smokers, COPD, or CF subjects obtained during lung transplantation was fixed in formalin and embedded in paraffin. Sections of bronchial tissue from mild asthmatics was kindly provided by Dr. James Gern (University of Wisconsin) and has been described previously[2]Five micron thick sections were used for detection of FoxO3A by confocal immunofluorescence microscopy (see below).

Immortalized human bronchial epithelial cell line (16HBE14o- cells) (obtained from Dr. Dieter Gruenert, University of California, San Francisco) was cultured, as described earlier [3], in MEM supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), glutamine and 10% (v/v) fetal bovine serum.

ELISA. Conditioned basolateral medium from cell cultures or lung homogenate supernatant were used to determine the protein levels of chemokines by ELISA (R&D systems, Minneapolis, MN) as described previously [1 4].

Transfection of 16HBE14o- cells. 16HBE14o- cells were reverse transfected with non-targetting (NT) or FoxO3A siRNA using Lipofectamine siRNAMAX (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The cells were either transfected with 5, 10 or 20 picomoles of non-targeting (NT) or FoxO3A siRNA (Sense strand 5' GGCUCCUCCUUGUACUCAATT 3' Antisense strand 5' UUGAGUACAAGGAGGAGCCTG 3') (Dharmacon, Inc., Chicago, IL) and incubated for 24 h. Media was changed and further incubated for 2 days and IL-8 protein levels in the cell culture conditioned medium were estimated by ELISA. Cells were lysed in RIPA buffer and cell lysates used for Western blot analysis to determine FoxO3A expression.

Isolation of nuclear, cytoplasmic and whole cell extracts. Nuclear and cytoplasmic protein extracts were prepared as described previously with some modifications[5]. In brief, the cells were incubated in 10mM HEPES buffer containing 10mM KCl, 10 mM EDTA, 1mM DTT, complete protease inhibitors (Roche, Indianapolis, IN) 1% IGEPAL on ice for 10 min. Cells were scrapped off of the plate and centrifuged. The supernatant containing cytoplasmic proteins was stored and the pellet was suspended in 20mM HEPES buffer containing , 0.4M NaCl, 10% glycerol, 1mM DTT and complete protease inhibitors, incubated on ice for 2h and centrifuged. The supernatant containing nuclear proteins was collected and stored at -70° C until analysis. Total cell lysates were prepared by lysing cells in RIPA buffer containing complete protease

inhibitors and phosphatase inhibitors, sodium fluoride and sodium orthovanadate as described previously [6].

Western blot analysis. Aliquots of whole cell, cytoplasmic or nuclear extracts containing equal amounts of total proteins [4] were resolved by 10% SDS- polyacrylamide gel electrophoresis, proteins transferred to nitrocellulose membrane and probed with antibodies to total and phosphoFoxO3A, phosphoAKT, total AKT (all three antibodies from Cell Signalling Technology, Inc., Boston, MA), phospho and total EGFR (Millipore, Bellerica, MA). To detect FoxO3A in mice antibody was purchased from Santa Cruze Biotechnology Inc. Bound antibody was detected by appropriate second antibody conjugated with horse radish peroxidase and chemiluminescent substrate. Specific bands were quantified by densitometry using NIH image J and normalized to β-actin or respective total proteins.

Immunodetection of FoxO3Aand IL-8 in bronchial biopsies. Paraffin sections of normal or COPD bronchi or mucociliary-differentiated primary AEC were deparaffinized , and subjected to antigen-retrieval treatment [7]. Sections were blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and incubated overnight at 4°C with rabbit polyclonal antibody to FoxO3A or IL-8 (R & D systems, Minneapolis, MN). Bound antibody was detected by second antibody conjugated to Alexafluor 598 or 488 (Invitrogen), counter stained with DAPI and visualized under confocal microscopy (Carl Zeiss, Thornwood, NJ).

Animals and treatment. Normal eight to ten week-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) maintained in specific pathogen free environment were exposed to elastase and LPS for four consecutive weeks as described previously [4 7]. One day after the last exposure to LPS, mice were orally gavaged with 0.3 ml of 0.5mg/ml quercetin or propylene glycol (vehicle) once a day for 10 days. With this method we achieved quercetin plasma levels of 0.2 μM and this was sufficient to inhibit lung inflammation in elastase/LPS-exposed mice [4].

Mice were then sacrificed, lungs collected and homogenized in PBS containing protease inhibitors and used for preparation of nuclear or cytoplasmic extracts or whole cell extracts. All experiments described herein were approved by the Animal Care and Use Committee of the University of Michigan.

In some experiments, mice were exposed to cigarette smoke or room air for 2h per day, 5 days a week for 6 weeks as described previously. The mean concentration of particulates collected during a 2-h exposure was 9.28 ± 1.45 mg. Under these conditions, animals developed mild emphysema with recruitment of moncytic cells to peripheral lungs [8]. Mice exposed to room air served as controls. During the last two weeks of cigarette smoke or room air- exposure mice were treated with either vehicle or quercetin.

Statistical analysis. Results are expressed as means ± SD. Data were analyzed by using SigmaStat statistical software (Systat Software, Inc., San Jose, CA). One-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc analysis was performed to compare more than two groups. To compare two groups, an unpaired t test with Welch's correction was used. A 'p' value <0.05 was considered significant.

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Supplemental Figure Legends

Supplemental Figure 1. Histology of normal and COPD airway epithelial cell cultures. Passage one cells from tracheobronchial segments of normal or COPD patients were grown at air/liquid interface and cells along with the membrane was fixed and embedded in paraffin. Cross sections were stained with H and E (A) or Periodic acid Schiff (PAS) reagent (B). Arrows in B represent PAS positive cells. Images are representative of cultures from 3 normal and 3 COPD subjects.

Supplemental Figure 2. Cigarette smoke-exposed mice do not show reduction in nuclear FoxO3A. Mice were exposed to cigarette smoke or room air (control) for 6 weeks and treated with quercetin or vehicle during 5^{th} and 6^{th} weeks. Mice were sacrificed, lungs were homogenized in PBS and divided into two equal portions. From one portion of lung homogenates nuclear extracts were prepared and FoxO3A levels determined by Western blot analysis (A) and the band intensities were analyzed by densitometry and expressed as fold increase over β-actin (B). Another portion of the lungs was centrifuged and supernatant used for ELISA to determine KC, MIP-2 and MCP-1 levles (C to E). Image represents nuclear FoxO3A from 3 mice per groups. Data represent mean±SD (n=5, different from room air-exposed animals treated with vehicle, $p \leq 0.05$; #different from cigarette smoke-exposed mice treated with vehicle $p \leq 0.05$, ANOVA with Tukey-Kramer post hoc analysis).

Supplemental Table 1. Characteristics of COPD and normal subjects

Number	Age	Gender	Smoking history	Emphysema	FEV1	FVC
	FEV1/FVC	Asthma	A1 antitrypsin			
			(pack·years)		(% predicted)	(% predicted)
COPD						
6	65	male	120	severe	15	1.95 (49)
	0.22	No	MM			
7	50	female	70	severe	17	1.89 (57)
	0.22	Yes	MM			
11	59	male	25	moderate	22	2.51 (56)
	0.28	No	MZ			
14	48	female	42	mild	17	2.12 (52)
	0.33	No	MM			
15	62	female	40	Severe	17	1.91 (55)
	0.31	No	MS			
16	77	male	50	Severe	20	2.35 (57)
	0.35	Yes	MM			
Normal donors						
1	54	female				
2	59	male				
6	50	female				
9	46	male				
25	51	male				
26	68	female				

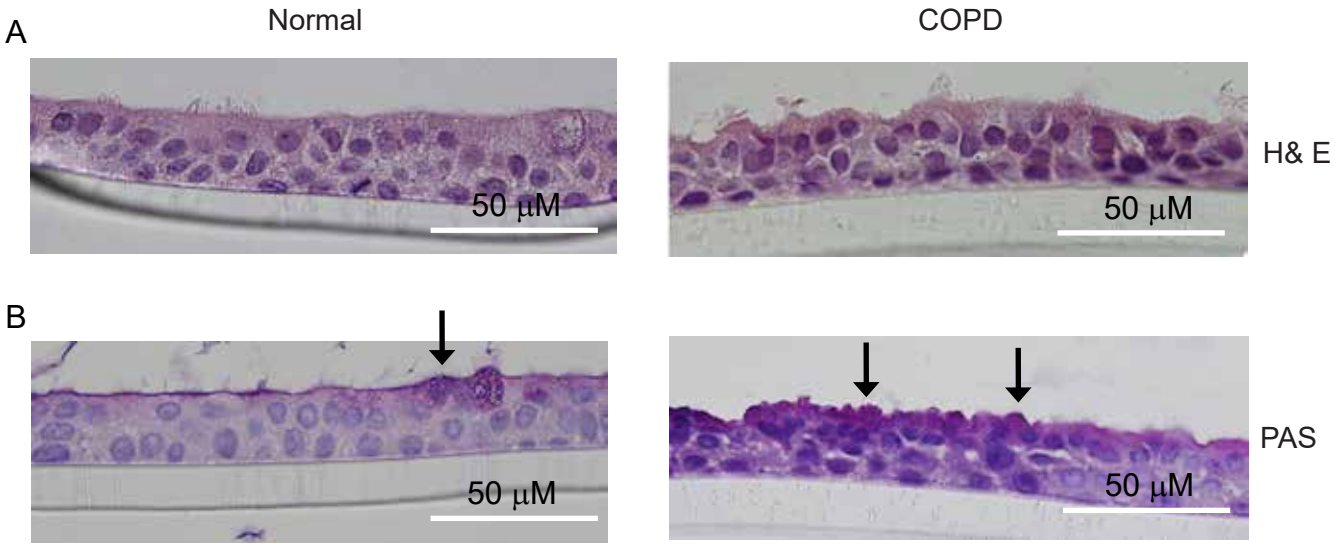
Tissue donor number designated as COPD 6,7 and 11 and normal 1, 2, 6, and 9 were obtained from the University of Michigan and have been previously described (1). Rest pf the tissues were obtained from the National Disease Research Interchange, Philadelphi, PA. Emphysema scores were based on visual reading of CT scans by radiologist.

Supplemental Table 2. Medication history of COPD patients

Number	Medication history
C6	LAMA, ICS, LABA, SABA, PPI, Montelukast, Tetracycline, Cartia, ASA
C7	LAMA, ICS, LABA, SABA, PPI, Montelukast, Fosamax
C11	LAMA, SABA, Allegra, ASA, HCTZ, Lotensin
C14	LAMA, SABA, PPI, glipizide
C15	LAMA, ICS, LABA, SABA, PPI, statins
C16	LAMA, LABA, Montelukast, statins

LAMA, long-acting muscarinic antagonist; ICS, inhaled corticosicoids; LABA, long-acting beta agonist; SABA, short-acting β2 agonists; PPI, proton pump inhibitor; ASA, acetylsalicylic acid; HCTZ, hydrochlorothiazide

Supplemental Figure 1



Supplemental Figure 2