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

1 *Primary cells and bronchial sections.* This study was approved by the University of
2 Michigan Investigational Review Board. All bronchial segments were obtained from intact and
3 healthy tissue of healthy non-smokers, smokers without COPD or from COPD subjects during
4 lung transplantation. None of the donors had exacerbation at the time of tissue collection. Five
5 out of 6 met symptomatic criteria for chronic bronchitis, whereas 4 had at least one symptomatic
6 exacerbation in the previous year. Tissues were obtained from either University of Michigan (4
7 healthy non-smokers, 2 healthy smokers and 3 COPD subjects) or National Disease Research
8 Interchange (Philadelphia, PA)) (tissues from 3 COPD and 2 healthy non-smokers). Airway
9 epithelial cells (AEC) isolated tracheobronchial segments of COPD or normal donors were
10 isolated and cultured at passage one as described earlier [1]. Briefly, airway epithelial cells at
11 passage 1 were cultured in transwells under submerged conditions for a week or until confluent.
12 Then, the cells were lifted to air-liquid interface to promote differentiation into mucociliary
13 phenotype. In some experiments, COPD cells differentiated into mucociliary phenotype were
14 treated with 1 µM erlotinib, 1 µM LY294002 (both from Cayman Chemical, Ann Arbor, MI), or
15 1 µM quercetin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or DMSO (vehicle control)
16 for 2 weeks. Transwells were shifted to new plate, containing fresh media and incubated for 24
17 h. Basolateral medium was collected for IL-8 determination and cells were used for protein
18 analysis by Western blotting.

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

1 Immortalized human bronchial epithelial cell line (16HBE14o- cells) (obtained from Dr.
2 Dieter Gruenert, University of California, San Francisco) was cultured, as described earlier [3], in
3 MEM supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), glutamine and
4 10% (v/v) fetal bovine serum.

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

8 *Transfection of 16HBE14o- cells.* 16HBE14o- cells were reverse transfected with non-
9 targetting (NT) or FoxO3A siRNA using Lipofectamine siRNAMAX (Invitrogen, Carlsbad, CA)
10 following manufacturer's instructions. The cells were either transfected with 5, 10 or 20
11 picomoles of non-targeting (NT) or FoxO3A siRNA (Sense strand 5'
12 GGCUCCUCCUUGUACUCAATT 3' Antisense strand 5' UUGAGUACAAGGAGGAGCCTG
13 3') (Dharmacon, Inc., Chicago, IL) and incubated for 24 h. Media was changed and further
14 incubated for 2 days and IL-8 protein levels in the cell culture conditioned medium were
15 estimated by ELISA. Cells were lysed in RIPA buffer and cell lysates used for Western blot
16 analysis to determine FoxO3A expression.

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

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

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

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

20 *Animals and treatment*. Normal eight to ten week-old C57BL/6 mice (Charles River
21 Laboratories, Wilmington, MA) maintained in specific pathogen free environment were exposed
22 to elastase and LPS for four consecutive weeks as described previously [4 7]. One day after the last
23 exposure to LPS, mice were orally gavaged with 0.3 ml of 0.5mg/ml quercetin or propylene
24 glycol (vehicle) once a day for 10 days. With this method we achieved quercetin plasma levels of
25 0.2 μM and this was sufficient to inhibit lung inflammation in elastase/LPS-exposed mice [4].

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

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

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

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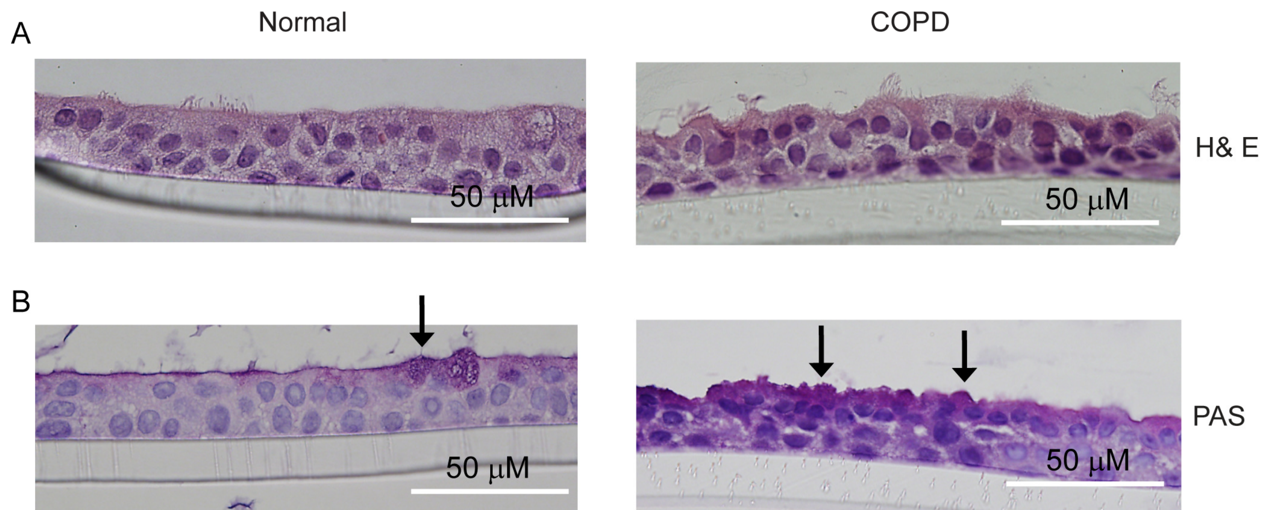
Supplemental Figure 1. Histology of normal and COPD airway epithelial cell cultures. Passage one cells from tracheobronchial segments of normal or COPD patients were grown at air/liquid interface and cells along with the membrane was fixed and embedded in paraffin. Cross sections were stained with H 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.

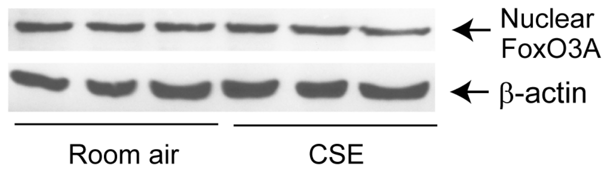
Supplemental Figure 1



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 5th and 6th 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 Figure 2

A



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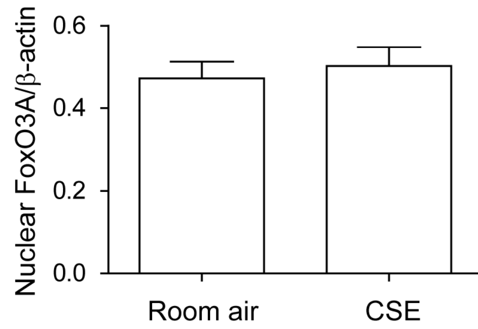

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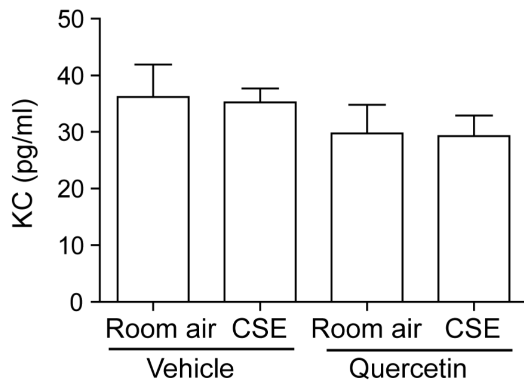

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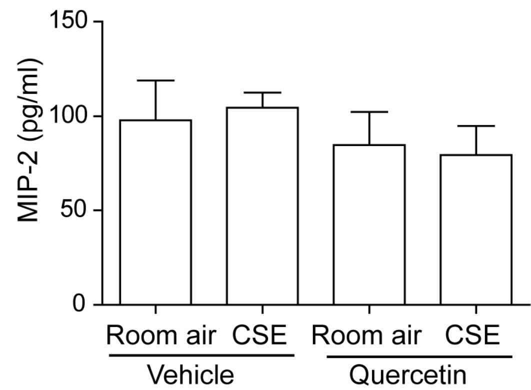

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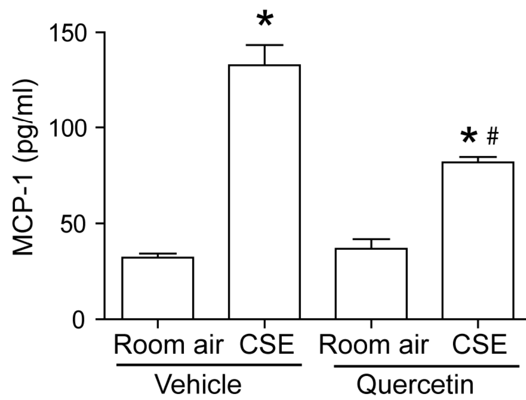

Supplemental Table 1. Characteristics of COPD and normal subjects

Number	Age	Gender	Smoking history (pack·years)	Emphysema	FEV1 (% predicted)	FVC (% predicted)	FEV1/FVC	Asthma	A1 antitrypsin
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