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

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Abstract

Background: Decreased activity of forkhead transcription factor class O (FoxO)3A, a negative regulator of NF- κ B-mediated chemokine expression, is implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD). Previously, we showed that quercetin reduces lung inflammation in a murine model of COPD. Here, we examined the mechanisms underlying decreased FoxO3A activation and its modulation by quercetin in COPD human airway epithelial cells and in a COPD mouse model.

Methods: Primary COPD and normal human airway epithelial cells were treated with quercetin, LY294002 or erlotinib for two weeks. IL-8 was measured by ELISA. FoxO3A, Akt, and EGF receptor (EGFR) phosphorylation and nuclear FoxO3A levels were determined by Western blot analysis. Effects of quercetin on lung chemokine expression, nuclear FoxO3A levels and phosphorylation of EGFR and Akt were determined in COPD mouse model.

Results: Compared to normal, COPD cells showed significantly increased IL-8, which negatively correlated with nuclear FoxO3A levels. COPD bronchial biopsies also showed reduced nuclear FoxO3A. Decreased FoxO3A in COPD cells was associated with increased phosphorylation of EGFR, Akt and FoxO3A and treatment with quercetin, LY294002 or erlotinib increased nuclear FoxO3A and decreased IL-8 and phosphorylation of Akt, EGFR and FoxO3A. Compared to control, elastase/LPS-exposed mice showed decreased nuclear FoxO3A, increased chemokines and phosphorylation of EGFR and Akt. Treatment with quercetin partially reversed these changes.

Conclusions: In COPD airways, aberrant EGFR activity increases PI 3-kinase/Akt-mediated phosphorylation of FoxO3A, thereby decreasing nuclear FoxO3A and increasing chemokine

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expression. Quercetin restores nuclear FoxO3A and reduces chemokine expression partly by modulating EGFR/PI 3-kinase/Akt activity.

Keywords

Akt; inflammation; quercetin; IL-8; PI-3 kinase

INTRODUCTION

Airways inflammation is one of the central features of chronic obstructive pulmonary disease (COPD). Airway epithelial cells (AEC) significantly contribute to inflammation by producing cytokines and chemokines in response to various stimuli, including cigarette smoke (CS). This in turn leads to recruitment of inflammatory cells to the airways. Under normal conditions, the expression of cytokines is tightly regulated by negative regulatory molecules, thereby limiting the influx of inflammatory cells and restoring the tissue homeostasis. Therefore, inactivation or reduced expression of negative regulatory molecules may result in sustained cytokine expression and development of airways inflammation. Previously, we demonstrated that AEC isolated from COPD patients express significantly more C-X-C chemokines than normal cells ¹, suggesting that COPD cells are defective in regulating cytokine expression.

The forkhead transcription factor (Fox)O3A negatively regulates pro-inflammatory cytokine expression ², in addition to playing a critical role in a wide variety of cellular processes, including immune regulation, and promoting resistance to oxidative stress ³⁴. FoxO3A inhibits IL-8 expression in cells under oxidative stress by sequestering the RelA/p65 component of NF- κ B, a transcription factor that stimulates IL-8 promoter activity ². Recently, FoxO3A deficiency was shown to increase susceptibility to CS-induced lung inflammation in mice, but the mechanisms are not well understood ⁵.

The regulation of FoxO3A activity is complex and primarily depends on post-translation modifications, such as phosphorylation, acetylation and ubiquitination, which affect its stability and subcellular localization (nuclear versus cytoplasmic) ⁶. Akt, a downstream target of PI-3 kinase, phosphorylates and promotes cytoplasmic accumulation of FoxO3A, ultimately leading to ubiquitination and degradation ⁷. FoxO3A that has been phosphorylated by Akt also binds to the 14-3-3 chaperone and remains in the cytoplasm ⁸. Phosphorylation of FoxO3A by Akt is also promoted by the acetylation of nuclear FoxO3A by CREB binding protein combined with decreased SIRT1 (a type II histone deacetylase), which results in exclusion of FoxO3A from the nucleus ⁹¹⁰. These observations suggest a critical role for Akt in regulation of FoxO3A nuclear localization, but that role has not been established in AEC.

Increased Akt phosphorylation is seen in bronchial tissue of smokers ¹¹ and CS activates PI-3 kinase activity via EGFR activation ¹²¹³. Phosphorylation of both EGFR and Akt is increased by oxidative stress, which has been implicated in the pathogenesis of COPD ¹⁴¹⁵. Endotoxin, a cell wall component of gram-negative bacteria that is present in appreciable amounts in CS ¹⁶¹⁷, also increases Akt phosphorylation via EGFR activation ¹⁸. Based on these observations, we hypothesized that activation of the EGFR/PI 3-kinase/Akt signaling

pathway decreases nuclear FoxO3A levels in COPD AEC, thereby increasing expression of pro-inflammatory cytokines such as IL-8.

Quercetin (3,3',4',5,7-pentahydroxyflavone), a plant flavanoid is a potent antioxidant. Quercetin also modulates activities of several lipid kinases, tyrosine kinases and serine/threonine kinases^{19–22}. Previously, we demonstrated that quercetin decreases chemokine levels and overall lung inflammation, and prevents progression of emphysema in mice displaying typical pathological and physiological features of COPD^{23,24}, but the mechanisms are not understood. The present study examines the mechanisms underlying the decreased nuclear FoxO3A levels in COPD AEC and whether quercetin decreases chemokine expression by restoring nuclear FoxO3A levels. In this study, we also examined whether quercetin reduces chemokine expression *in vivo* by modulating nuclear FoxO3A levels.

METHODS (Details provided in on-line supplement)

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. Characteristics of COPD and normal donors are provided in Supplemental Table 1 and 2. AEC were isolated and cultured at air-liquid interface to promote differentiation into mucociliary phenotype as described¹. In some experiments, COPD mucociliary-differentiated AEC were treated with 1 μ M erlotinib, LY294002 or quercetin for 2 weeks.

Bronchial tissue from healthy non-smokers, COPD or CF subjects obtained during lung transplantation was fixed in formalin and embedded in paraffin.

ELISA.

Conditioned basolateral media or lung homogenate supernatants were used to quantify chemokine levels by ELISA¹²³.

Transfection of 16HBE14o- cells.

Immortalized 16HBE14o- AECs were reverse transfected with non-targeting (NT)- or FoxO3A-siRNA and incubated for 2 days²⁵. Cell culture medium was used for IL-8 determination and cells for expression of FoxO3A protein.

Western blot analysis of nuclear, cytoplasmic and whole cell extracts.

Nuclear and cytoplasmic protein extracts and total cell extracts were prepared as described previously^{25,26}. Aliquots of whole cell, cytoplasmic or nuclear extracts containing equal amounts of total proteins were subjected to Western blot analysis and specific bands quantified by densitometry^{23,25}.

Immunodetection of FoxO3A and IL-8.

Paraffin sections were deparaffinized, and subjected to immunofluorescence staining using antibody to FoxO3A as described previously²⁴ and/or IL-8 (R & D Systems, Minneapolis, MN) and sections were visualized under confocal microscopy (Carl Zeiss, Thornwood, NJ).

Animals and treatment.

Normal 8–10 week-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were exposed to elastase and LPS for four consecutive weeks and treated with quercetin or propylene glycol (vehicle), as described previously^{23,24}. Mice were exposed to cigarette smoke or room air for 6 weeks and treated with quercetin or vehicle during the last two weeks. Mice were then euthanized, and lung homogenates were used to prepare nuclear, cytoplasmic or whole cell extracts. All experiments described herein were approved by the Animal Care and Use Committee of the University of Michigan.

Statistical analysis.

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

RESULTS

Reduced Nuclear FoxO3A correlates with elevated IL-8 expression in AEC.

Both normal and COPD AEC grown at air/liquid interface showed mucocilliary differentiation (Supplemental Figure 1A) and transepithelial resistance of 400 to 600 $\Omega \cdot \text{cm}^2$. Compared to normal, COPD cultures also showed more PAS positive cells (Supplemental Figure 1B). Immunofluorescence microscopy indicated that while normal cells show FoxO3A primarily in the nucleus, COPD AEC show majority of FoxO3A in the cytoplasm (Figure 1A). Consistent with this, Western blot analysis showed significantly less nuclear FoxO3A in COPD AEC than in normal (Figure 1B and 1C). In contrast, there was no difference in nuclear FoxO3A levels between AEC from healthy smokers and normal, therefore subsequent experiments were performed with only normal and COPD cells. Despite reduced nuclear FoxO3A, COPD AEC showed total FOXO3A levels similar to normal AEC (Figure 1D and 1E). In addition, COPD AEC showed increased IL-8 protein compared to normal AEC as observed previously¹ (Figure 2A). Importantly, nuclear FoxO3A levels showed a strong negative correlation with IL-8 protein between individual subjects (Figure 2B). By immunofluorescence microscopy, IL-8 protein was observed in almost all cells in the cultures indicating that IL-8 expression is not specific to one cell type (Figure 2C), however COPD AEC showed comparatively more IL-8 protein than normal AEC.

To exclude the possibility that the observed difference between normal and COPD AEC in nuclear FoxO3A was simply due to *in vitro* culturing conditions, we also immune-localized FoxO3A in bronchial sections obtained from COPD or normal subjects. In normal bronchial

sections, majority of FoxO3A was found to co-localize with the nucleus of bronchial epithelial cells (Figure 3A), but in COPD sections, FoxO3A was mostly found in the cytoplasm and rarely in the nucleus. In both COPD and normal bronchial biopsies, IL-8 protein was observed in almost all the cells of bronchial epithelium, but the intensity was higher in COPD (Figure 3B). These results indicate that cultured AEC truly represents the changes in FoxO3A distribution and IL-8 expression observed *in vivo*.

To determine whether FoxO3A regulates IL-8 expression in AEC, we knocked-down FoxO3A using siRNA and measured basal IL-8 expression in immortalized AEC, because primary AEC are not readily amenable to transfection. Compared to NT siRNA-, FoxO3A siRNA-transfected cells showed significantly increased levels of IL-8 (Figure 4A). FoxO3A siRNA-, but not NT siRNA-transfected cells showed complete knockdown of FoxO3A expression (Figure 4B). These results confirmed that FoxO3A regulates basal IL-8 expression in human AEC.

Akt phosphorylation is elevated in COPD AEC.

Next, we examined whether reduced nuclear FoxO3A in unstimulated COPD AEC is due to increased phosphorylation. Cytoplasmic extracts from COPD AEC showed significantly increased phosphorylated FoxO3A compared to normal AEC (Figure 5A and 5B). Because Akt, a downstream target of PI-3 kinase phosphorylates FoxO3A, we examined Akt phosphorylation and the effects of PI-3 kinase inhibitor, LY294002 on nuclear FoxO3A. Compared to normal, COPD AEC showed increased Akt phosphorylation (Figure 5C and 5D) and treatment with LY294002 decreased phosphorylation of both Akt and FoxO3A, increased nuclear FoxO3A (Figure 5E), and decreased IL-8 expression in COPD AEC (Figure 5F). These observations imply that increased PI-3 Kinase/Akt activation is largely responsible for the observed nuclear FoxO3A reduction in COPD AEC.

Increased EGFR activity correlates with Akt phosphorylation in COPD AEC.

PI-3 kinase/Akt signaling pathway can be activated via tyrosine kinases including growth factor receptors²⁷. Since EGFR activity is upregulated in COPD patients²⁸, and cigarette smoke increases EGFR activity¹²¹³, we next looked for evidence of elevated EGFR activity in COPD AEC. EGFR activity, as assessed by EGFR phosphorylation on tyrosine residue 1173, was higher in COPD than in normal AEC (Figure 6A and 6B). Treatment with erlotinib, an inhibitor of EGFR decreased EGFR, Akt and FoxO3A phosphorylation, increased nuclear FoxO3A (Figure 6C) and reduced IL-8 protein (Figure 6D) in COPD AEC, implying that upregulated EGFR may be responsible for PI-3 kinase/Akt activation and subsequent decrease in nuclear FoxO3A and increased basal IL-8 levels in COPD AEC.

Quercetin treatment decreases IL-8 expression by inhibiting EGFR and Akt activation in COPD cells.

One of the ways by which the anti-inflammatory nutraceutical quercetin appears to work is by blocking both tyrosine and threonine/serine protein kinases¹⁹⁻²². We examined whether quercetin decreases IL-8 secretion in COPD AEC by reducing phosphorylation of EGFR, Akt and FoxO3A. Compared to DMSO-treated cells, COPD AEC treated with quercetin showed decreased IL-8 levels (Figure 7A), reduced phosphorylation of EGFR and Akt, and

increased nuclear FoxO3A (Figure 7B). Increased nuclear FoxO3A in quercetin treated COPD AEC was further confirmed by immunolocalization (Figure 7C). Thus quercetin decreases IL-8 levels in COPD AEC in association with decreased activities of both EGFR and PI-3 kinase.

Quercetin treatment increases nuclear FoxO3A levels in elastase/LPS-exposed mice.

To determine whether these findings were relevant *in vivo*, we examined our established murine model²³²⁴²⁶ for changes in nuclear FoxO3A, and for the phosphorylation state of EGFR and Akt. Compared to PBS-exposed mice, elastase/LPS-exposed mice showed a significantly increased phosphorylation of both EGFR (Figure 8A and8B) and Akt (Figure 8C and8D) and reduced nuclear FoxO3A (Figure 8E and8F). Due to the unavailability of suitable antibody to mouse phospho-FoxO3A, we could not examine FoxO3A phosphorylation. Quercetin treatment significantly reduced phosphorylation of EGFR and Akt and increased nuclear FoxO3A in elastase/LPS-exposed mice (Figure 9A) and also decreased the KC and MIP-2 to the levels that observe in PBS-exposed mice(Figure 9B and9C).

We also examined the effect of quercetin in CS-induced changes in FoxO3A distribution in mice. Interestingly, CS-exposed mice showed nuclear FoxO3A, and KC and MIP-2 levels similar to room air-exposed mice (Supplemental Figure 2A to 2D) and not surprisingly quercetin did not affect nuclear FoxO3A, KC or MIP-2 levels. In contrast, compared to room air-exposed, CS-exposed mice showed significant increase in MCP-1 levels and quercetin treatment reduced MCP-1 levels in CS-exposed mice (Supplemental Figure 2C), indicating that quercetin as expected negatively regulate chemokines whose expression is NF- κ B independent.

Nuclear FoxO3A levels are decreased in bronchial epithelium of CF patients.

We determined the FoxO3A distribution in sections of bronchial biopsies from CF patients (who also show chronic lung inflammation) by immune-localization. Bronchial epithelium showed FoxO3A primarily in cytoplasm and rarely in the nucleus (Figure 10) similar to that observed in COPD bronchial biopsies.

DISCUSSION

The principal findings of this study support a biochemical network in AECs of subjects with COPD by which persistent activation of the receptor tyrosine kinase EGFR mediates increased airway inflammation via activation of PI-3 kinase and Akt, consequent cytoplasmic retention of the anti-inflammatory transcription factor FoxO3A and increased basal levels of important neutrophil chemo-attractant IL-8. Further, we show that low concentrations of quercetin, a non-toxic plant flavanol with anti-oxidant and kinase-inhibiting properties, block this pathway both in differentiated COPD AEC *in vitro* and in a murine model²³²⁴²⁶ that faithfully mimics many features of COPD pathology and physiology. These novel findings provide a mechanistic explanation for our previous observation of excessive C-X-C chemokine elaboration in COPD¹, and motivate additional

study of a quercetin as a therapy to mitigate the development or progression of oxidative stress-induced lung damage.

These results complement and extend recent evidence for an important regulatory role for FoxO3A in COPD pathogenesis in humans⁵, as it has in other chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis²⁹³⁰. FoxO3A-deficient mice develop spontaneous, multi-systemic inflammatory syndrome, accompanied by NF- κ B hyperactivity and increased pro-inflammatory cytokines². FoxO3A-deficient mice are also more susceptible to CS-induced lung inflammation and airspace enlargement⁵. We extend the previous finding that Foxo3A expression is downregulated in lung epithelial cells of COPD patients⁵, by showing reduced nuclear FoxO3A localization, which we confirmed in bronchial epithelium of COPD subjects *in situ*, and by demonstrating increased phosphorylated cytoplasmic FoxO3A in AEC from COPD patients. Interestingly, we found a similar decrease in nuclear FoxO3A in elastase/LPS-exposed mice, in which we have previously shown pathological changes typical of COPD including emphysema, extensive inflammation, remodeling of small airways, goblet cell hyperplasia, and susceptibility to viral and bacterial infections²³²⁴²⁶. In contrast, CS-exposed mice showed no changes in nuclear FoxO3A similar to that observed in AEC from healthy smokers, indicating the requirement for other endogenous/exogenous factors in addition to CS to reduce nuclear FoxO3A levels. These findings may partly explain why not all smokers develop COPD. Because FoxO3A interacts with RelA/p65 component of NF- κ B in the nucleus, thereby inhibiting NF- κ B binding to IL-8 promoter⁵, it is not surprising that we observed increased IL-8 expression in COPD AEC. Increased IL-8 secretion has also been noted in CF airways and the observation that nuclear FoxO3A is decreased in bronchial epithelium of these patients indicate that reduction in nuclear FoxO3A is not specific to COPD, but rather associated with chronic inflammatory conditions. Additional studies will be needed to define the possible consequences of altering nuclear FoxO3A levels on elaboration of other inflammatory mediators in the resting state and in response to relevant pro-inflammatory stimuli or infection. Further, clinical studies are required to establish the direct relationship of reduced nuclear FoxO3A or FoxO3A polymorphisms with disease severity in COPD patients.

Given the importance of FoxO3A shuttling between the nucleus and cytoplasm based on its phosphorylation state¹⁰, identification of Akt and PI-3 kinase as mediators of FoxO3A regulation in AEC is important to a greater understanding of smoking or other environmental factors-induced lung disease. Akt phosphorylation is increased in the lungs and peripheral blood monocytes of COPD patients compared to normal subjects³¹ and also increased by CS in both mice and in human AEC¹¹³². Although CS can increase PI-3 kinase activity either by decreasing the expression of PTEN (phosphatase and tensin homologue deleted on chromosome 10)³³ or via increasing the activation of EGFR, we believe that the latter may be more relevant mechanism, because there was no decrease in the expression of PTEN in COPD AEC compared to normal (data not shown). In contrast, our finding of significantly increased EGFR phosphorylation with unchanged total EGFR levels in COPD cells compared to normal, and results using erlotinib, which inhibits EGFR specifically, both implicate EGFR activity for increased PI-3 kinase activity in COPD cells. Consistent with our observations, EGFR activity is increased in COPD patients²⁸ and CS stimulates PI-3

kinase/Akt activity via EGFR activation *in vitro*¹²¹³. Moreover CS also increases LPS-stimulated EGFR activity in normal AEC³⁴. Although the epigenetic changes that maintains elevated EGFR activation in COPD AECs are not known at present, this study indicate the suitability of COPD AECs for understanding the biochemical mechanisms that mediates increased airway inflammation in COPD.

Quercetin has a broad spectrum of anti-inflammatory actions, dependent at least in part on its ability to block the ATP-binding sites of multiple lipid and protein kinases. Previously, we have demonstrated that quercetin decreases TNF- α -stimulated IL-8 production by blocking PI-3 kinase activity in human airway epithelial cells³⁵ and significantly reduces chemokine expression and lung inflammation in elastase/LPS-exposed mice²³²⁴. We interpret the current data on the effect of quercetin on nuclear FoxO3A localization as further support for a EGFR-PI-3 kinase-Akt pathway that ultimately regulates production of inflammatory mediators. However, one cannot rule out the possibility that quercetin may also increase nuclear FoxO3A levels by increasing SIRT1 levels, as we and others have shown that quercetin increases expression of SIRT1²³³⁶. SIRT1 deacetylates nuclear FoxO3A, particularly in cells undergoing oxidative stress, which show increased nuclear accumulation of protein acetylases, including CBP/ p300-associated factor⁹. Deacetylation by SIRT1 prevents phosphorylation of FoxO3A by Akt and subsequent export of FoxO3A from nucleus to cytoplasm.

In conclusion, our studies provide an insight into one of the mechanisms by which IL-8 expression is upregulated in COPD airways in the stable state. We demonstrate that COPD AEC cultured *in vitro* retain their pro-inflammatory phenotype, with sustained increases in activity of EGFR and Akt, which in turn modulate nuclear FoxO3A levels. We show that low dose of quercetin modulates the activities of EGFR and Akt, and inhibits chemokine expression both *in vitro* and *in vivo*. These results together with our previous studies²³ suggest that treatment with quercetin may reduce inflammation and improve lung function in COPD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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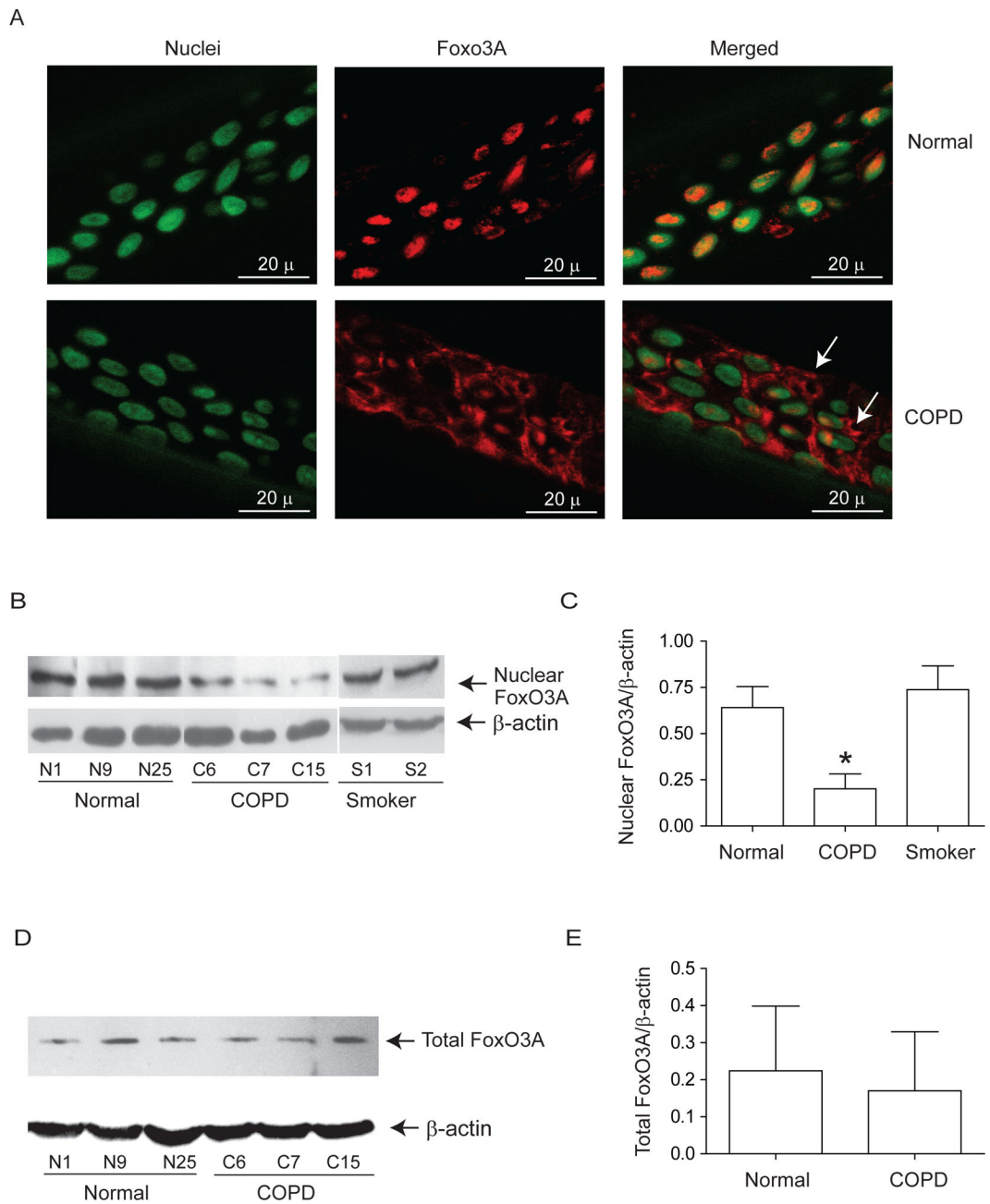


Figure 1.

Nuclear FoxO3A is decreased in COPD AEC under basal conditions. Primary AEC from COPD or normal subjects were cultured in transwells at air/liquid interface to promote differentiation into mucociliary phenotype. (A) Cultures were fixed and embedded in paraffin. Five micron thick sections were deparaffinized, and incubated with antibody to FoxO3A. Bound antibody was detected by second antibody conjugated with Alexafluor-598 (red) and nuclei were counterstained with DAPI (green). Co-localization of FoxO3A with nuclei appears yellow and arrows point to cytoplasmic FoxO3A. Images are representative of AEC obtained from 3 COPD and 3 normal subjects. (B to C) Nuclear extracts from well-differentiated AEC cultures from healthy non-smokers, healthy smokers and patients with

COPD were examined for FoxO3A by Western blot analysis and band densities were quantified and expressed as fold increase over β -actin. (D and E) Total proteins from healthy non-smokers or COPD AEC were subjected to Western blot analysis with antibody to FoxO3A and levels of FoxO3A was normalized to β -actin. Data in C to E represents mean \pm SD calculated from cells isolated from three to six subjects per group; *, different from normal cells, p 0.05, ANOVA.

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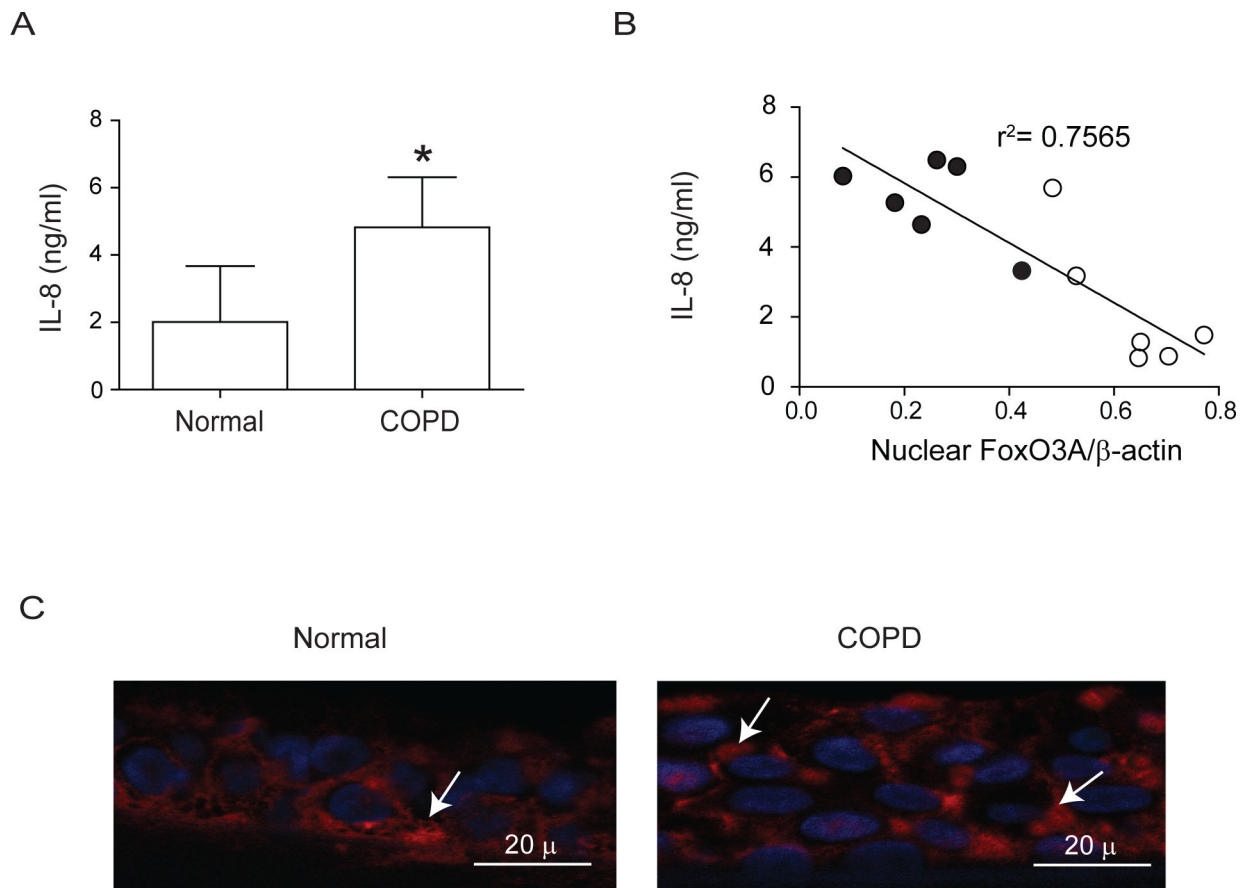


Figure 2.

IL-8 levels negatively correlates with nuclear FoxO3A. (A) Basolateral media from unstimulated COPD and normal AEC differentiated into mucociliary phenotype was used to determine IL-8 by ELISA. Data represents mean \pm SD calculated from cells isolated from six subjects per group; *, different from normal cells, $p < 0.05$; unpaired t test. (B) Correlation between nuclear FoxO3A (from Figure 1C) and IL-8 was assessed by linear regression analysis (○ and ● represent normal and COPD AEC respectively). (C) Sections of COPD and normal AEC were probed with antibody to IL-8, bound antibody was detected by antimouse IgG labeled with Alexafluor-598 and sections were counterstained with DAPI to visualize nuclei. Arrows represents IL-8. Images are representative of 3 normal and 3 COPD cultures.

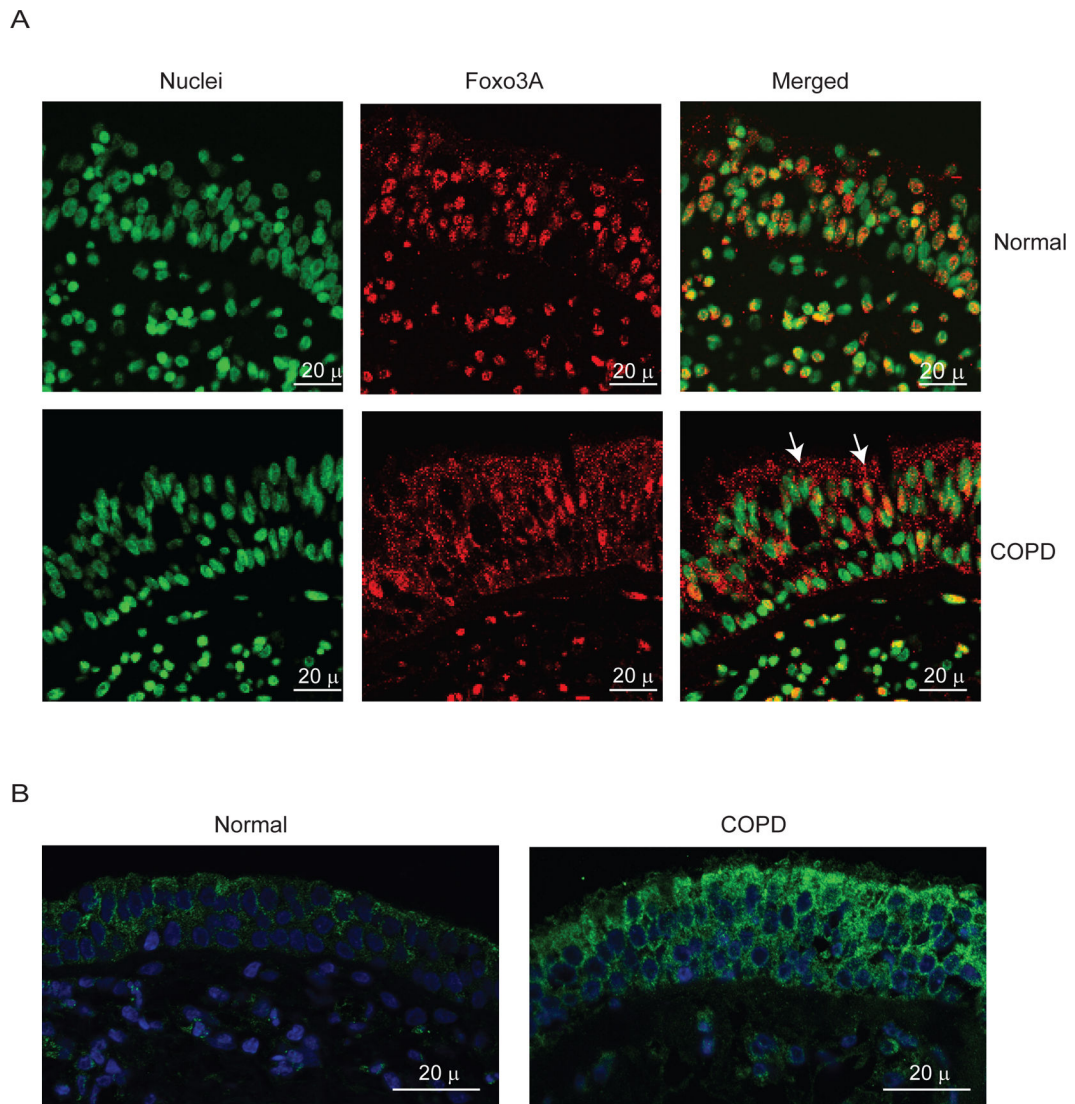


Figure 3.

COPD bronchial epithelium shows decreased nuclear FoxO3A and increased IL-8 expression. Paraffin sections of bronchial tissue from COPD or normal subjects were deparaffinized, blocked and incubated with antibody to FoxO3A or IL-8. Bound antibodies were detected by antimouse or antirabbit IgG conjugated with Alexafluor-594 and nuclei were counterstained with DAPI. (A) FoxO3A and nuclei are represented by red and green respectively; co-localization of FoxO3A with nuclei appears yellow; arrows show FoxO3A in the cytoplasm. (B) IL-8 and nuclei are represented by green and blue respectively. Images are representative of bronchial sections from three COPD and two normal subjects.

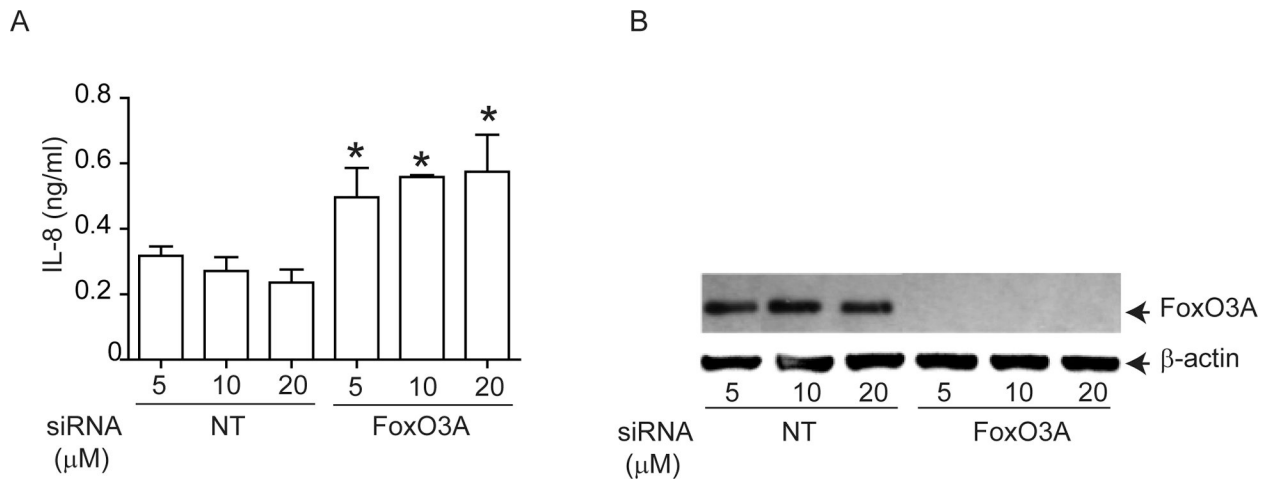
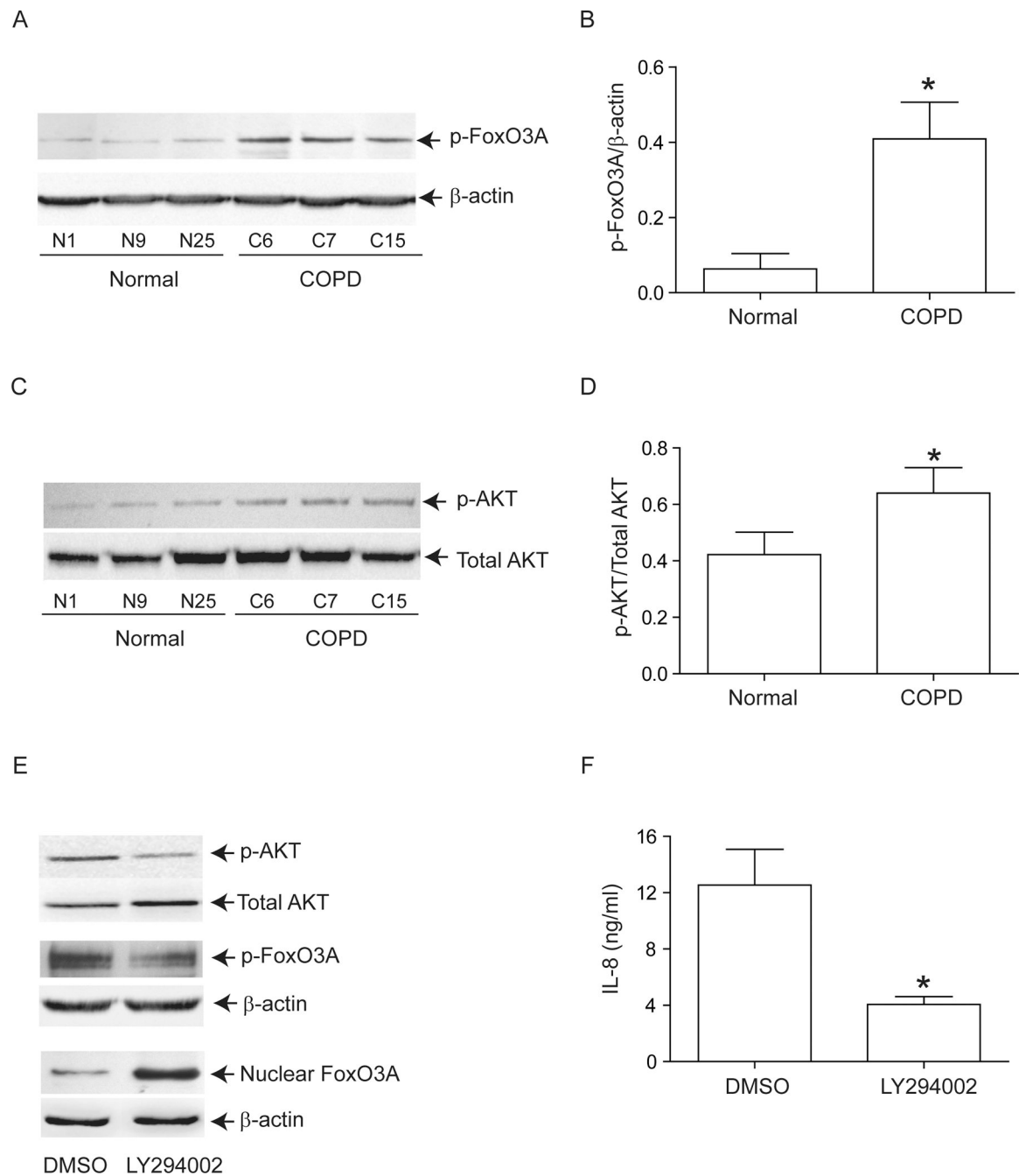


Figure 4.

Genetic inhibition of FoxO3A reduces IL-8 expression in normal airway epithelial cells. 16HBE14o- cells were reverse transfected with non-targeting (NT)- or FoxO3A-specific siRNA. Cells were incubated for 48 h, then media was changed and incubated for a further 24 h before culture supernatants were harvested and total cellular protein was isolated. (A) IL-8 levels measured by ELISA. Data represent mean and average from three independent experiments (*, different from cells transfected with NT siRNA, $p < 0.05$, unpaired t test). (B) FoxO3A protein levels by Western blot analysis. Representative of three independent experiments.

**Figure 5.**

Inhibition of PI-3 kinase increases nuclear FoxO3A and decreased IL-8 in COPD AEC. Primary AEC from COPD subjects or normal volunteers were cultured as described in Figure 1, then (A, B) cytoplasmic extracts were subjected to Western blot analysis with antibody to phospho-FoxO3A; (C, D) total cell lysates were subjected to Western blot analysis with antibodies to phospho-Akt and total Akt and band densities were quantified by NIH image J. Data in B and D represents AEC from 6 subjects per group. (E) Mucociliary-differentiated COPD AEC were treated with DMSO (vehicle control) or 1 μ M LY294002 for 2 weeks. Total proteins (for Akt), nuclear (for FoxO3A) or cytoplasmic (for

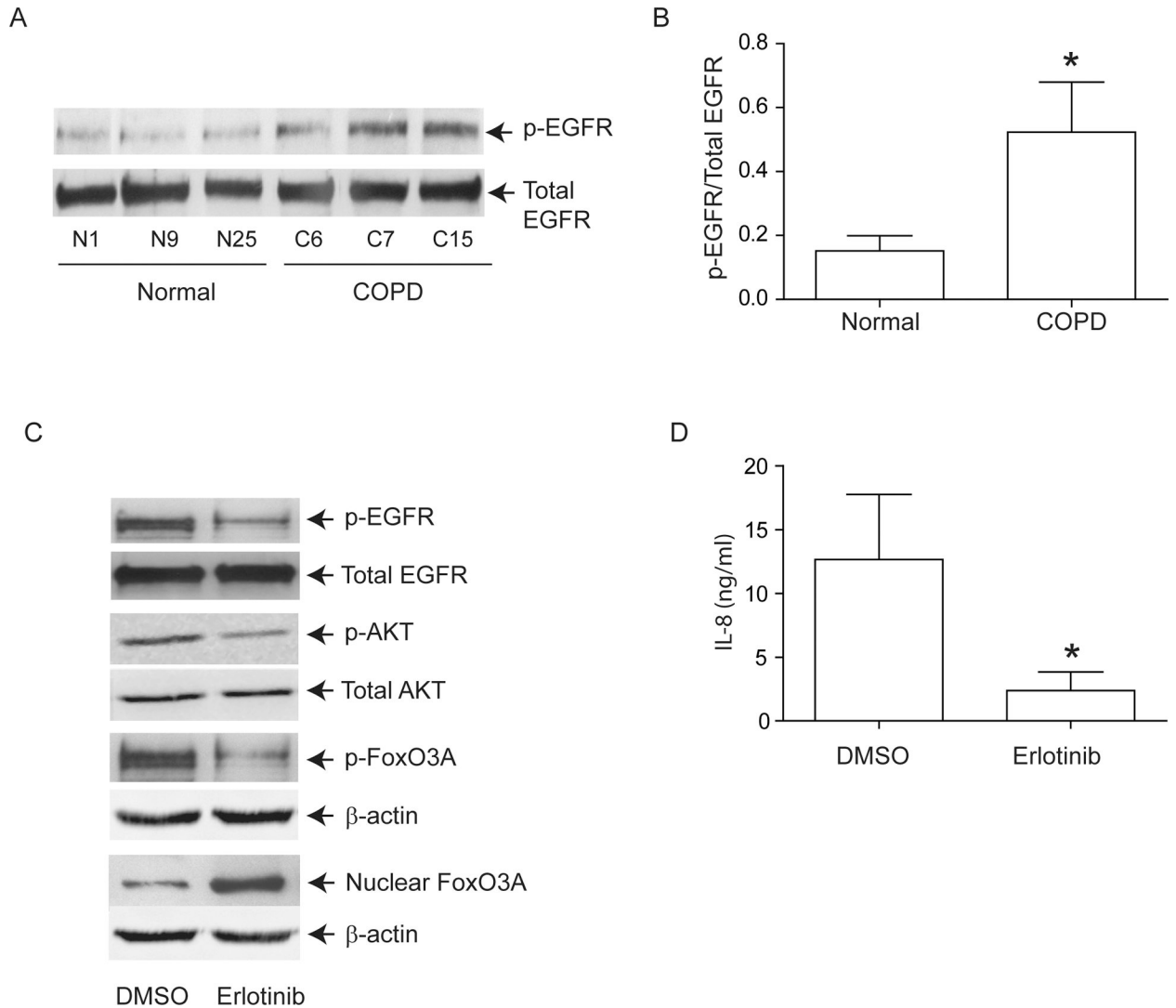
phospho-FoxO3A) extracts were isolated from cells and subjected to Western blot analysis with relevant antibodies. Images are representative of 3 independent experiments. (F) IL-8 levels in the basolateral medium of the cells determined by ELISA; Mean \pm SD from three independent experiments, performed in duplicates with cells obtained from three donors (*, different from normal airway epithelial cells, $p < 0.05$, unpaired t test).

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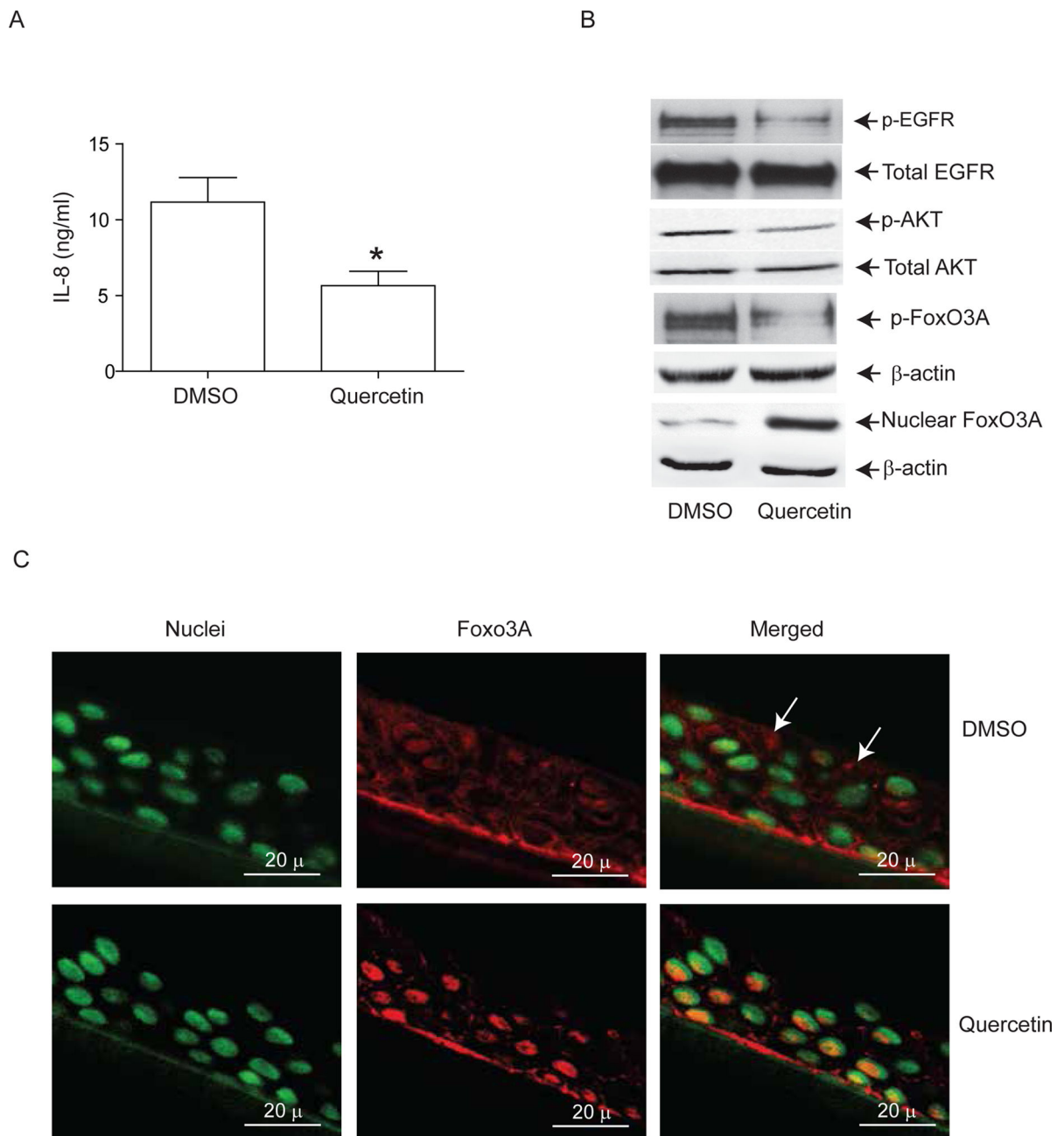
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**Figure 6.**

EGFR phosphorylation is increased in COPD cells and inhibition of EGFR decreases IL-8 and increases nuclear FoxO3A in these cells. Primary human AEC from COPD subjects or normal volunteers were cultured as described in Figure 1. (A, B) Total cell lysates were prepared and subjected to Western blot analysis using antibodies to phospho-EGFR and total EGFR. A, Representative western blot; B, phospho-EGFR/total EGFR ratios, $n = 6$ per group. (C) mucociliary-differentiated COPD cells were treated with DMSO (vehicle control) or 1 μ M erlotinib for 2 weeks. Total proteins (for EGFR), nuclear (for FoxO3A) or cytoplasmic (for phospho-FoxO3A) extracts were isolated from cells and subjected to Western blot analysis with relevant antibodies. Blots are representative of three independent experiments. (D) IL-8 levels in the basolateral medium were determined by ELISA. Data represents mean and SD calculated from 3 independent experiments performed in duplicates with cells obtained from three donors (* different from normal airway epithelial cells, $p < 0.05$, unpaired t test).



normal airway epithelial cells, $p < 0.05$, unpaired t test). (C) Some cultures were fixed and embedded in paraffin. Five micron thick sections were used for immune-localization of FoxO3A by confocal immunofluorescence microscopy. Co-localization of FoxO3A with nucleus appears yellow and arrows point to cytoplasmic FoxO3A. Images are representative of 3 independent experiments.

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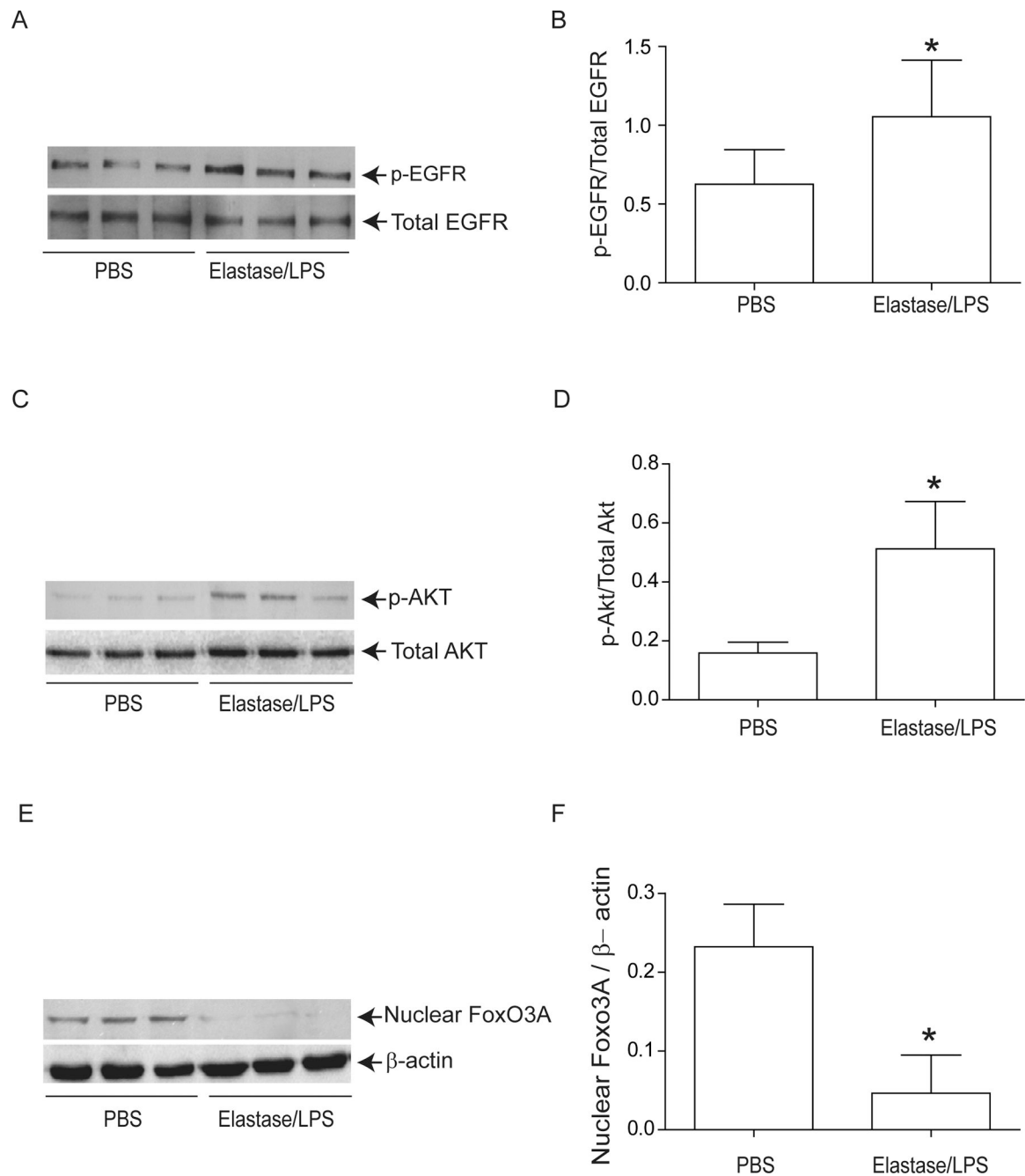


Figure 8.

Elastase/LPS-exposed mice show decreased nuclear FoxO3A levels and increased phosphorylation of EGFR and Akt. Mice were exposed to elastase/LPS or PBS for 4 weeks. Lungs were excised, homogenized in PBS and aliquots of homogenates were used for either total protein (for EGFR and Akt) or nuclear protein (for FoxO3A) extraction. Aliquots equivalent to equal protein was subjected to Western blot analysis. Western blot images in A, C, and E show samples analyzed from 3 individual animals. Panels B, D and E represent ratios of p-EGFR/total EGFR, p-Akt/total Akt and FoxO3A/ β -actin respectively. Data

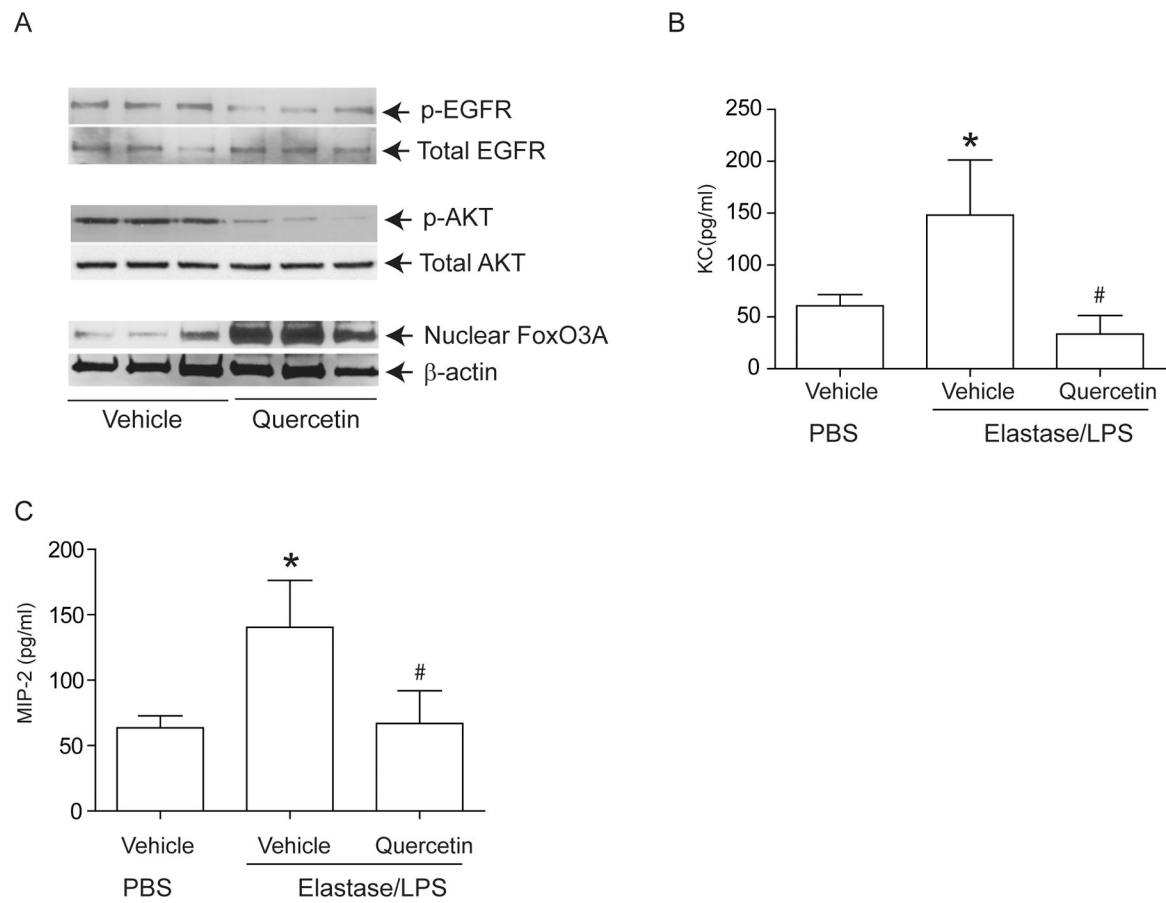
represents mean and SD calculated from 3 independent experiments (n=6 mice * different from PBS-exposed animals, p 0.05, unpaired t test).

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**Figure 9.**

Quercetin treatment reduced chemokine expression in Elastase/LPS-exposed mice correlates with increased nuclear FoxO3A levels. Mice were exposed to elastase/LPS for 4 weeks and then treated with quercetin or vehicle for 2 weeks. PBS-exposed animals treated with vehicle were used as controls in some experiments. Lungs were excised, homogenized in PBS and aliquots of homogenates were used for either nuclear (for FoxO3A), or total protein (for EGFR and Akt) extraction. Aliquots equivalent to equal protein was subjected to Western blot analysis. Western blot images in A, B, and C show samples analyzed from 3 individual mice. (B,C) Lung homogenate supernatants were used for determination of chemokines by ELISA. Data represents mean and SD calculated from 3 independent experiments (n=6-9 * different from PBS-exposed animals treated with vehicle, p 0.05; #different from elastase/LPS exposed mice treated with vehicle p 0.05, ANOVA with Tukey-Kramer post hoc analysis).

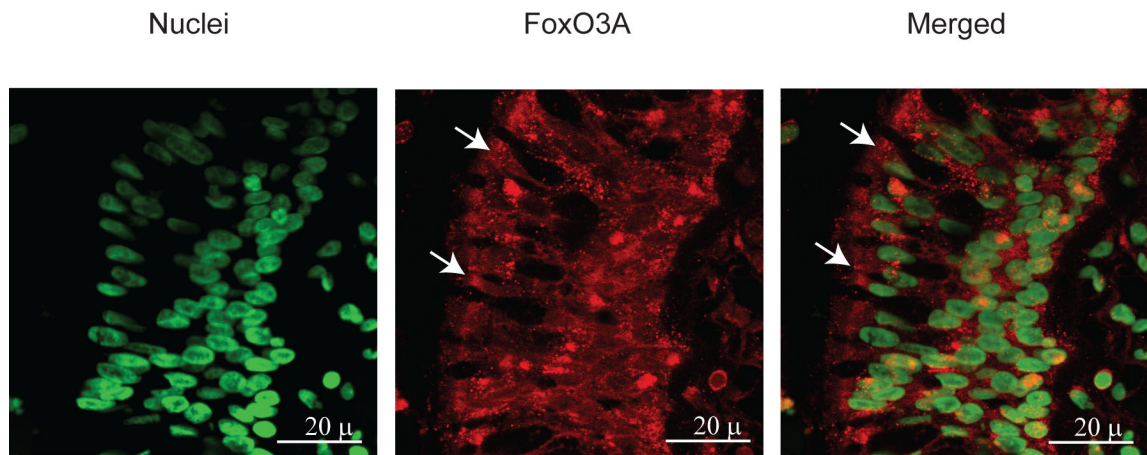


Figure 10.

Nuclear FoxO3A is decreased in CF bronchial epithelium. Paraffin section of bronchial tissue from CF patients was deparaffinized, blocked and incubated with antibody to FoxO3A. Bound antibody was detected by antirabbit IgG conjugated with Alexafluor-594 and nuclei were counterstained with DAPI. (A) FoxO3A and nuclei are represented by red and green respectively; co-localization of FoxO3A with nuclei appears yellow; arrows show FoxO3A in the cytoplasm. Images are representative of bronchial sections from three CF patients.