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Hypoxia-inducible factor-1 signalling promotes goblet cell hyperplasia in airway epithelium

Vasiliy V Polosukhin^{1,*}, Justin M Cates², William E Lawson^{1,5}, Aaron P Milstone¹, Anton G Matafonov², Pierre P Massion^{1,4,5}, Jae Woo Lee⁶, Scott H Randell^{7,8}, and Timothy S Blackwell^{1,3,4,5}

¹Department of Medicine, Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA

²Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN, USA

³Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN, USA

⁴Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN, USA

⁵Department of Veterans Affairs Medical Center, Nashville, TN, USA

⁶Department of Anesthesia and Perioperative Care, University of California, San Francisco, CA, USA

⁷Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, NC, USA

⁸Department of Cell and Molecular Physiology, University of North Carolina, Chapel Hill, NC, USA

Abstract

Goblet cell hyperplasia is a common feature of chronic obstructive pulmonary disease (COPD) airways, but the mechanisms that underlie this epithelial remodelling in COPD are not understood. Based on our previous finding of hypoxia-inducible factor-1 α (HIF-1 α) nuclear localization in large airways from patients with COPD, we investigated whether hypoxia-inducible signalling could influence the development of goblet cell hyperplasia. We evaluated large airway samples obtained from 18 lifelong non-smokers and 13 former smokers without COPD, and 45 former smokers with COPD. In these specimens, HIF-1 α nuclear staining occurred almost exclusively in COPD patients in areas of airway remodelling. In COPD patients, $93.2 \pm 3.9\%$ (range 65 – 100%) of goblet cells were HIF-1 α positive in areas of goblet cell hyperplasia, whereas nuclear HIF-1 α

*Correspondence to: Vasiliy V Polosukhin, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, T-1218 MCN, Nashville, TN 37232-2650, USA. vasiliy.v.polosukhin@vanderbilt.edu.

No conflicts of interest were declared.

Author contributions

VVP, JMC, WEL, SHR and TSB contributed to the conception and/or design of the study; VVP and AGM conducted experiments and performed data analyses; APM, PPM and JWL provided the clinical samples. All authors were involved in drafting and/or critical revision of the manuscript and approved the final submitted version.

SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:
Supplementary materials and methods.

was not detected in individuals without COPD or in normal-appearing pseudostratified epithelium from COPD patients. To determine the direct effects of hypoxia-inducible signalling on epithelial cell differentiation *in vitro*, human bronchial epithelial cells (HBECs) were grown in air-liquid interface cultures under hypoxia (1% O₂) or following treatment with a selective HIF-1 α stabilizer, (2*R*)-[(4-biphenylsulphonyl)amino]-*N*-hydroxy-3-phenyl-propionamide (BiPS). HBECs grown in hypoxia or with BiPS treatment were characterized by HIF-1 α activation, carbonic anhydrase IX expression, mucus-producing cell hyperplasia and increased expression of MUC5AC. Analysis of signal transduction pathways in cells with HIF-1 α activation showed increased ERK1/2 phosphorylation without activation of epidermal growth factor receptor, Ras, PI3K-Akt or STAT6. These data indicate an important effect of hypoxia-inducible signalling on airway epithelial cell differentiation and identify a new potential target to limit mucus production in COPD.

Keywords

bronchial epithelium; cell differentiation; chronic obstructive pulmonary disease; mucous cell hyperplasia; mucus

Introduction

Mucus hypersecretion is an important pathophysiological feature of chronic obstructive pulmonary disease (COPD). Excessive mucus in the airways in COPD is associated with increased frequency of infections, more rapid decline in pulmonary function and increased mortality [1,2]. In COPD, mucus hypersecretion results from hyperplasia of goblet cells in bronchial epithelium and hypertrophy of submucosal glands [3,4]. Goblet cell hyperplasia has been linked to several intracellular signalling pathways. The best-characterized inflammatory pathway that influences goblet cell hyperplasia is the Th2 lymphocyte-mediated mechanism involving interleukin-13 (IL-13) and signal transducer and activator of transcription 6 (STAT6) [5,6]. Other studies have indicated epidermal growth factor receptor (EGFR) tyrosine kinases in the induction of goblet cell hyperplasia via a Ras–Raf–MAPK/ERK kinase (MEK1/2)–extracellular signal-regulated kinase (ERK1/2) cascade [7,8]. Activation of phosphatidylinositol 3-kinase (PI3K) and Akt may also be involved in EGFR-mediated goblet cell hyperplasia [9]. A two-step model of chronic goblet cell hyperplasia after Sendai virus infection in mice demonstrated that EGFR activation in airway epithelial cells promotes ciliated cell hyperplasia, and the presence of IL-13 results in trans-differentiation of ciliated cells into goblet cells [9]. Despite this information regarding pathways that stimulate goblet cell hyperplasia, the relevant mechanisms that determine epithelial remodelling in humans with COPD are not well defined.

We have previously identified hypoxia-inducible signalling in bronchial epithelial cells in COPD patients [10]. Specifically, we showed stabilization and nuclear translocation of hypoxia-inducible factor-1 α (HIF-1 α) in bronchial epithelial cells in large airways from patients with severe COPD associated with progressive fibrous thickening of the subepithelial reticular basement membrane and reduction of subepithelial microvasculature. In addition, a conserved consensus motif in the MUC5AC gene promoter for transcriptional

HIF-1 has been demonstrated, suggesting possible involvement of hypoxia-inducible signalling in up-regulation of mucus production [11]. HIF-1 is a heterodimeric basic–helix–loop–helix–PAS transcription factor consisting of HIF-1 α and HIF-1 β subunits. Activity of HIF-1 is limited by availability of the HIF-1 α subunit, because HIF-1 α is actively degraded under normoxic conditions via oxygen-dependent hydroxylation of specific proline residues (Pro 402 and Pro 564) by prolyl-4-hydroxylases and interaction with the Von Hippel–Lindau (pVHL) tumour suppressor protein. In hypoxia, HIF-1 α stabilizes, translocates to the nucleus, dimerizes with HIF-1 β and activates transcription of target genes containing hypoxia-response elements within their promoter or enhancer [12]. Here, we investigated whether hypoxia-inducible signalling shifts airway epithelial cell differentiation towards goblet cell hyperplasia and contributes to mucus hypersecretion in COPD. This work helps to clarify the pathogenesis of mucus overproduction in COPD and identifies a new potential therapeutic target to limit mucus production.

Materials and methods

Clinical material

Lung tissue specimens containing large (segmental/subsegmental) bronchi were collected from lifetime non-smokers (never smokers) without known lung disease and former smokers with or without COPD (Table 1). Tissue specimens from 18 never smokers and 13 former smokers without COPD were obtained from donor lungs which were declined for lung transplantation. Tissue specimens from 22 patients with mild/moderate COPD (GOLD Stage I–II [13]) were obtained from lungs resected for solitary tumours, whereas tissue specimens from 23 patients with severe–very severe COPD (GOLD Stage III–IV [13]) were obtained from the explanted lungs of transplant recipients (Table 1). The study was approved by the Institutional Review Board at Vanderbilt University.

Histology and immunohistochemistry

Paraffin sections (5 μ m thick) were stained with haematoxylin and eosin (H&E) and the periodic acid–Schiff (PAS) reaction for detection of mucin. Immunohistochemistry (IHC) was performed on unstained sections with primary antibodies against MUC5AC (mouse monoclonal, clone CLH2, No. MAB2011; Millipore, Billerica, MA, USA) or HIF-1 α (rabbit polyclonal, clone H-206, No. sc-10 790; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a standard immunoperoxidase/avidin–biotin complex protocol (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA).

Air–liquid interface cultures

Three human bronchial epithelial cell (HBEC) lines were obtained from proximal bronchi of healthy non-smoking humans and cultured in an air–liquid interface (ALI) system, using Millicell culture plate inserts (12 mm; Millipore) [14]. Hypoxic conditions (1% oxygen, 5% carbon dioxide, 94% nitrogen) were created by placing cells in a sealed chamber (Billups–Rothenberg, Del Mar, CA, USA). For HIF-1 α stabilization under normal oxygen tension, (2*R*)-[(4-biphenylsulphonyl)amino]-*N*-hydroxy-3-phenylpropionamide (BiPS) was used [15]. BiPS was dissolved in dimethylsulphoxide (DMSO) and diluted 1 : 1000 in phosphate-buffered saline (PBS), pH 7.2–7.4, for a final concentration of 80 μ M. 50 μ l BiPS solution

was added apically on selected inserts for 2 h, followed by pipette removal twice daily. DMSO diluted in PBS (1 : 1000) served as vehicle control. The cells were grown in hypoxia or treated with BiPS during days 14–28 in ALI culture. At day 28, the cells were harvested and used for histological and protein analyses.

HBEC histology and immunohistochemistry

Cells were fixed in 4% paraformaldehyde in PBS for 2 h and then embedded in paraffin. Paraffin sections (5 μ m) were stained with H&E. IHC was performed for MUC5AC, HIF-1 α or carbonic anhydrase IX (CA IX, rabbit polyclonal antibody, clone H-120, No. sc-25 599; Santa Cruz Biotechnology), using the protocol for bronchial specimens.

Western blotting

Western blots (WB) of HBEC extracts were performed using rabbit polyclonal antibodies against β -actin (No. A2066; Sigma-Aldrich, St. Louis, MO, USA), HIF-1 α (No. NB100-479; Novus Biologicals, Littleton, CO, USA), total EGFR (clone 1005, No. sc-03; Santa Cruz Biotechnology) or the following antibodies from Cell Signalling Technology (Danvers, MA, USA): phosphorylated EGFR (Thr669, No. 3056; or Tyr845, No. 2231), phosphorylated p44/42 mitogen-activated protein kinase (p44/42 MAPK or ERK1/2, Thr202/Tyr204, No. 9101), total p44/42 MAPK (or total ERK1/2, No. 9102), serine–threonine kinase-phosphorylated Akt (Ser473, No. 4060), total Akt (No. 9272), phosphorylated STAT6 (Tyr641, No. 9361) or total STAT6 (No. 9362).

Ras activation assay

To detect Ras activation in treated cells, a Ras activation enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer's (Millipore) recommendations, using a Synergy-2 luminometer (BioTek Instruments, Winooski, VT, USA); see Supporting information, Supplementary methods. Data are represented as a percentage of positive control (EGF-stimulated HeLa cells).

MUC5AC ELISA

At day 28, the cells were thoroughly washed with PBS and apical washings were collected 24 h later. To detect MUC5AC protein in apical washings, we used a rabbit polyclonal anti-MUC5AC antibody (clone H-160, No. 20 118; Santa Cruz Biotechnology) and HRP (horseradish peroxidase)-conjugated goat anti-rabbit polyclonal antibody (No. 31 462; Thermo Scientific, Rockford, IL, USA). For the detailed protocol, see Supporting information, Supplementary methods.

Statistics

Statistical analysis was performed with GraphPad InStat (GraphPad Software, San Diego, CA, USA) using the Kruskal–Wallis test with a *post hoc* Dunn's multiple comparisons test. $p < 0.05$ was considered significant. The frequency of occurrence of goblet cell hyperplasia was compared using a χ^2 test.

Results

HIF-1 α staining correlates with MUC5AC expression in areas of goblet cell hyperplasia

In large airway specimens obtained from both never smokers and former smokers without COPD, the majority of the bronchial epithelium was pseudostratified, with a normal ratio of ciliated and goblet cells (1 : 8–20). Some of these samples contained isolated foci of goblet cell hyperplasia (Figure 1A). In contrast, a mosaic pattern of epithelial remodelling was present in tissue specimens obtained from patients with COPD, as we previously reported [10,16]. The most common structural appearance of the epithelium in these airways was goblet cell hyperplasia, which is characterized by a predominance of cells that are larger than normal surface columnar cells and whose cytoplasm is distended by numerous PAS- and MUC5AC-positive mucus granules (Figure 1B). Table 2 lists the distribution of normal-appearing epithelium and goblet cell hyperplasia for each group analysed.

As shown in Figure 1B, we found intense nuclear and cytoplasmic HIF-1 α staining in areas of goblet cell hyperplasia in COPD patients. In these areas of goblet cell hyperplasia, a large majority of goblet cells (mean 93.2%, 100% median, range 65–100%) were positive for nuclear HIF-1 α . While goblet cells were predominantly HIF-1 α -positive, other epithelial cells in areas of goblet cell hyperplasia (basal cells and ciliated epithelial cells) were much less likely to show HIF-1 α nuclear staining. In contrast, nuclear HIF-1 α staining was not detected in normal-appearing epithelium in any group or in areas of goblet cell hyperplasia in never smokers and former smokers without COPD. Since nuclear HIF-1 α expression was detected only in structurally abnormal bronchial epithelium in COPD patients, this association suggests involvement of hypoxia-inducible signalling in the induction of goblet cell hyperplasia in COPD. These findings also suggest that mechanisms underlying goblet cell hyperplasia may be different in individuals with COPD compared to those without COPD.

Hypoxia-inducible signalling affects HBEC differentiation *in vitro*

To further examine the relationship between hypoxia-inducible signalling and cell differentiation, we set up ALI cultures of isolated HBECs growing under normoxia (21% O₂), hypoxia (1% O₂) or with BiPS administration to stabilize HIF-1 α in normoxia. We observed that cells grown in normoxic conditions developed fully differentiated pseudostratified ciliated epithelium. Under hypoxic conditions, however, the epithelial layer assumed a pseudostratified appearance with a reduction in fully differentiated ciliated cells and an increased proportion of MUC5AC-positive cells situated between columnar surface cells. In BiPS treated cultures, there was an even more dramatic phenotype with almost complete replacement of ciliated cells by MUC5AC-positive cells (Figure 2A, B). Analysis of apical washings demonstrated significant increases of MUC5AC protein concentrations in hypoxic or BiPS-treated cell cultures compared to untreated or vehicle-treated cell cultures (Figure 2C). Similar results were obtained from all three HBEC lines.

To detect hypoxia-inducible signalling in HBECs, HIF-1 α expression was analysed by IHC and WB (Figure 3). IHC analysis showed nuclear translocation of HIF-1 α following exposure to hypoxia or BiPS (Figure 3A). Furthermore, increased accumulation of HIF-1 α

was detected in cell lysates by WB (Figure 3B, C). We also analysed expression of CA IX, a marker of HIF-1 α -dependent gene activation. CA IX is a transmembrane protein that catalyses the reversible hydration of CO₂ to H₂CO₃, thus regulating pH in hypoxic cells [17]. CA IX was only detected in hypoxic conditions or following BiPS treatment, thus confirming HIF-1 α activation (Figure 3D). Cells treated with vehicle only during 14–28 days of culture had structural organization identical to that of untreated cells and did not show HIF-1 α stabilization/translocation or CA IX expression (not shown).

ERK1/2 is activated by hypoxia-inducible signalling

Phosphorylation of the HIF-1 α subunit is an important step in its stabilization and activation [18,19]. Since ERK1/2 is involved in hypoxia-induced HIF-1 α activation and ERK1/2 inhibitors can impair HIF-1 α stabilization [18], we evaluated ERK1/2 activation in HBECs under hypoxic conditions or following BiPS treatment. As shown in Figure 4, we detected an increase of phospho-ERK1/2 and the ratio of phospho-ERK1/2 to total cellular ERK1/2 in hypoxic or BiPS-treated HBECs compared to normoxic cells or cells treated with vehicle control. This finding indicates that ERK1/2 activation occurs downstream of HIF-1 α and may be involved in goblet cell hyperplasia.

EGFR-mediated signalling can also result in ERK1/2 activation and is considered an important pathway regulating goblet cell hyperplasia [7,8]. Therefore, we investigated whether EGFR-mediated signalling is induced by hypoxia or HIF-1 α activation, potentially explaining ERK1/2 activation and altered epithelial cell development under these conditions. To identify activated EGFR, phosphorylation of EGFR at Thr669 or Tyr845 was analysed by WB. Both Thr669 and Tyr845 are major phosphorylation sites after EGF stimulation and function in maintenance of the activated EGFR kinase, providing a binding surface for substrate proteins [20]. By WB, we found no evidence of EGFR phosphorylation in HBECs exposed to hypoxia (Figure 5A) or BiPS (Figure 5B) compared to normoxic conditions or vehicle controls, respectively. We also evaluated Ras activation, since Ras is an important downstream transducer of EGFR signalling. No increase in activated Ras was identified in hypoxic HBECs (Figure 5C) or BiPS-treated HBECs (Figure 5D), consistent with the lack of phosphorylated EGFR found in these studies.

PI3K-Akt activation is another mechanism of the EGFR-mediated signalling that can influence epithelial cell differentiation [9]. To investigate this pathway, we evaluated Akt phosphorylation in HBECs under hypoxic conditions or BiPS treatment. Slightly increased phospho-Akt was observed in hypoxic cells (Figure 5E) and decreased phospho-Akt was detected in BiPS-treated cells (Figure 5F). Together, these studies suggest that hypoxia-induced goblet cell hyperplasia is not mediated by EGFR and its downstream signalling pathways.

Since Th2 type inflammation may also stimulate goblet cell hyperplasia in airway epithelium via IL-13R-mediated STAT6 signalling [5,6], we asked whether hypoxia-inducible signalling might affect epithelial cell remodelling by activating STAT6. In order to investigate this possibility, phosphorylation of STAT6 was analysed in HBECs treated with hypoxia or BiPS. No phosphorylation of STAT6 was identified in either hypoxic HBECs

(Figure 5G) or BiPS-treated HBECs (Figure 5H), indicating that this pathway is not activated in these cells under hypoxic conditions or HIF-1 α activation.

Discussion

In this study, we demonstrated that hypoxia-inducible signalling skews airway epithelial cell differentiation towards goblet cell hyperplasia. In humans with COPD, we detected HIF-1 α nuclear expression in airway epithelial cells in areas of goblet cell hyperplasia and up-regulation of MUC5AC expression. *In vitro* experiments demonstrated that both hypoxia and pharmacological HIF-1 α stabilization induce mucous cell hyperplasia in isolated HBECs. Interestingly, epithelial remodelling under hypoxic conditions was characterized by moderate mucous cell hyperplasia, whereas treatment with BiPS resulted in more pronounced HIF-1 α activation and marked mucous cell hyperplasia, suggesting a dose-dependent effect of HIF-1 α activity on epithelial differentiation. At the same time, both hypoxia and BiPS treatments were characterized by increased MUC5AC protein concentrations in apical washings. We also showed that mucous cell hyperplasia initiated by hypoxia-inducible signalling is accompanied by increased ERK1/2 phosphorylation without evidence of EGFR pathway activation or STAT6 activation. In combination, these studies indicate that HIF-1 α directly promotes mucous cell hyperplasia and suggest that this mechanism could be important in COPD.

In contrast to COPD airways, goblet cell hyperplasia was much less common in never smokers and former smokers without COPD and was not associated with HIF-1 α activation. Since the median duration of smoking cessation was similar in former smokers without COPD and those with COPD, it appears unlikely that the increased frequency of goblet cell hyperplasia and HIF-1 α activation in COPD are related to the direct effects of tobacco smoke. Although we cannot rule out the effects of ageing and cumulative smoke exposure on these end points (since COPD patients in our study were older and had more pack years of smoking), the most likely explanation for our findings is that HIF-1 α activation results from the airway remodelling characteristic of COPD and contributes to altered epithelial cell differentiation. The role of HIF-1 α appears to be relatively selective for COPD, and other mechanisms may drive goblet cell hyperplasia in other settings, including non-smokers and former smokers without COPD.

Although the underlying mechanisms activating HIF-1 α in COPD are uncertain, activation of HIF-1 α may occur secondary to: (a) hypoxia-initiated HIF-1 α stabilization; (b) induction of HIF-1 α mRNA and protein synthesis to such a degree that classical degradative pathways are overwhelmed; (c) modification of HIF-1 α by phosphorylation to prevent its degradation; and/or (d) phosphorylation and inactivation of prolyl hydroxylases that hydroxylate HIF-1 α [11]. We have previously shown that large airway remodelling in COPD is accompanied by significant thickening and fibrosis of the subepithelial basement membrane, reduction of subepithelial microvasculature and substantial perivascular fibrosis [16]. These processes may significantly reduce oxygenation of the airway mucosa, leading to activation of hypoxia-inducible signalling in airway epithelial cells in COPD. Although somewhat surprising given the proximity of airway epithelium to the relatively high oxygen tension of the airway, the finding of HIF-1 α activation in areas of airway remodelling suggests that

diffusion of oxygen across the airway epithelial barrier may be limited. However, other factors besides low oxygen tension may affect HIF-1 α activation in COPD airways.

In addition to hypoxia, HIF-1 α may be stabilized and activated in response to growth factors and pro-inflammatory cytokines, such as EGF, tumour necrosis factor- α (TNF α) and IL-1 β [21 – 24], vascular hormones [25,26] or viral proteins [27,28]. The mechanism of HIF-1 α stabilization and activation under normoxic conditions is strikingly different from those under hypoxia. Whereas tissue hypoxia is associated with protein stabilization and increased half-life, normoxic activation occurs via increased HIF-1 α protein synthesis or its modification [11,23,24]. For example, it has been demonstrated that stimulation of primary HBECs by pro-inflammatory cytokines TNF α /IL-4 induces expression and transcriptional activity of HIF-1 α under normoxic condition and amplifies HIF-1 α activation in hypoxia [22]. Since the inflammatory microenvironment in COPD is enriched with cytokines and pro-inflammatory mediators [29], this could be another mechanism of HIF-1 α stabilization/activation in COPD.

Phosphorylation plays a key role in HIF-1 α regulation [19,30,31]. For example, ERK1/2-dependent phosphorylation of HIF-1 α on Ser641 and Ser643 is required for its nuclear translocation and transcriptional activation [32]. Moreover, selective inhibition of ERK1/2 is able to impair HIF-1 α activation but does not inhibit its DNA binding activity [19]. Since the ERK1/2 MAP kinase pathway is activated in bronchial epithelial cells in chronic bronchitis and COPD [33,34], this pathway might also contribute to HIF-1 α activation in airway epithelium via its phosphorylation and stabilization. Interestingly, we observed that phosphorylation of ERK1/2 is increased in HBECs after exposure to hypoxia or BiPS treatment. Therefore, activation of ERK1/2 in epithelium under BiPS treatment may result in a positive feedback loop for HIF-1 α activation.

The presence of a conserved consensus motif in the MUC5AC promoter for HIF-1 α in airway epithelial cells suggests a direct up-regulation of mucus production by hypoxia-inducible signalling [11]. Moreover, HIF-1 α may up-regulate other genes important for goblet cell differentiation and function, such as the trefoil factor family [35]. At the same time, increased ERK1/2 activation by hypoxia-inducible signalling may indicate an additional (indirect) mechanism of goblet cell hyperplasia through activation of the MAPK-mediated cascade.

Identification of pathways responsible for goblet cell hyperplasia and mucus hypersecretion is important for understanding COPD pathogenesis. We speculate that mucus overproduction in COPD is based on an altered epithelial cell microenvironment and induction of hypoxia-inducible signalling in affected epithelial cells. Initially, tobacco smoke exposure, infectious agents or other environmental factors cause epithelial cell damage, followed by release of pro-inflammatory cytokines and growth factors by injured epithelial cells [29]. These mediators induce airway inflammation and fibroblast and myofibroblast activation, leading to fibrous remodelling of the subepithelium [36,37]. We believe that progressive remodelling of subepithelial connective tissue and reduced microvasculature in turn affect the bronchial epithelium microenvironment and create conditions for initiation of hypoxia-inducible signalling in affected epithelial cells [10], resulting in goblet cell hyperplasia even in the

absence of ongoing cigarette smoke injury. Additionally, growth factors and pro-inflammatory cytokines may contribute to stabilization and activation of HIF-1 α [21,22], affecting epithelial cell differentiation in individuals with COPD. Thus, our results suggest that hypoxia-inducible signalling is a potential therapeutic target to prevent progression of epithelial structural abnormalities and mucus overproduction in COPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Hypoxia-inducible signalling is detected in areas of goblet cell hyperplasia in large airways of patients with COPD. (A) Representative images of bronchial epithelium from former smokers without COPD; top row, pseudostratified ciliated bronchial epithelium with normal ratio of basal, ciliated and goblet cells; bottom row, pseudostratified bronchial epithelium with hyperplasia of goblet cells. (B) Representative images of bronchial epithelium from patients with COPD; top row, normal-appearing pseudostratified ciliated bronchial epithelium with normal ratio of basal, ciliated and goblet cells; bottom row, pseudostratified bronchial epithelium with hyperplasia of goblet cells. There was a significant increase of goblet cell size and an approximate doubling of the height of the epithelial layer in COPD compared to former smokers without COPD. Column I, H & E-stained tissue sections; column II, PAS-stained tissue sections; column III, IHC with anti-MUC5AC antibody (brown stain); MUC5AC-positive granules were detected in single cells in normal-appearing pseudostratified epithelium (top) and in goblet cells with hyperplasia (bottom); column IV, IHC with anti-HIF-1α antibody. Cytoplasmic detection of HIF-1α was rarely observed in epithelial cells in former smokers without COPD and in normal-appearing bronchial epithelium in patients with COPD, while nuclear detection was observed in goblet cell hyperplasia in COPD. All images, $\times 400$ magnification.

Figure 2.

Hypoxia and HIF-1 α stabilization affect HBEC differentiation towards mucus-producing cell hyperplasia *in vitro*. (A) H & E-stained paraffin sections. Normal-appearing pseudostratified ciliated epithelium was noted in untreated control cultures compared to hyperplasia of mucus-producing cells in both hypoxic and BiPS-treated cultures. (B) IHC with anti-MUC5AC antibody (brown stain). Increased proportion of MUC5AC-positive cells situated between columnar surface cells in hypoxia- and BiPS-treated cultures. All images $\times 600$ magnification. (C) Graph showing MUC5AC protein concentrations in apical washings. * $p < 0.01$ compared to untreated control (normoxia); ** $p < 0.01$ compared to vehicle-treated control.

Figure 3.

Hypoxia and BiPS treatments *in vitro* are characterized by HIF-1α stabilization and nuclear translocation and CA IX expression. (A) IHC of HBEC ALI cultures using anti-HIF-1α antibody. (B) IHC of HBEC ALI cultures using anti-CA IX antibody. Nuclear HIF-1α staining and cytoplasmic CA IX staining were observed only in hypoxic and BiPS-treated cultures. All images, ×600 magnification. (C, D) Western blots for HIF-1α from HBEC lysates obtained from cells grown under hypoxic conditions compared to normoxic controls (C) or BiPS treatment compared to vehicle-treated controls (D), normalized to β-actin.

Figure 4.

WB analysis of ERK1/2 activation under hypoxia (A) and BiPS treatment (B) showing significant increase in ERK1/2 phosphorylation. Densitometry analysis of ERK1/2 phosphorylation by western blots reveals a significant increase in the ratio of phosphorylated ERK1/2 (p-ERK1/2) to total ERK1/2 (t-ERK1/2) after hypoxia exposure (C) or BiPS treatment (D). Data are mean $\pm$ standard error of the mean (SEM) of three cell lines in duplicate. * $p < 0.01$ versus control.

Figure 5.

Analysis of EGFR, Ras, Akt and STAT6 activation. (A, B) WB analysis of EGFR after hypoxia (A) or BiPS (B) treatments, showing no evidence of increased phosphorylation relative to normoxia or vehicle. PC, positive control (A549 cells treated with EGF for 15 min); p-EGFR, phosphorylated EGFR; t-EGFR, total EGFR; Thr669 and Tyr845, sites of EGFR phosphorylation. (C, D) Ras activation assay after hypoxia (C) or BiPS (D) treatments, presented as a percentage of Ras activity in positive control (supplied by manufacturer). Data are mean $\pm$ SEM of three cell lines in duplicate. (E, F) WB analysis of Akt activation after hypoxia (E) or BiPS (F) treatments; p-Akt, phosphorylated Akt; t-Akt, total Akt. (G, H) WB analysis of STAT6 after hypoxia (G) or BiPS (H) treatments. PC, positive control (human lymphocytes treated with IL-13 for 15 min); p-STAT6, phosphorylated STAT6; t-STAT6, total STAT6.

Table 1

Clinical characteristics of study participants *

	Never smokers Never smokers without COPD	Former smokers Former smokers without COPD	COPD patients by GOLD criteria (former smokers)	
			COPD I-II	COPD III-IV
Number of study participants	18	13	22	23
FEV ₁ (% of predicted)	–	–	72 (56 – 90)	21 (17 – 42)
FEV ₁ /FVC (% of FVC)	–	–	0.58 (0.41 - 0.69)	0.34 (0.21 - 0.53)
Age (years)	54.5 (17 - 70)	54.0 (37 - 76)	65.5 (58 - 80)	55.0 (43 - 65)
Sex				
Male	8	9	12	12
Female	10	4	10	11
Smoking history				
Packs/year	–	40.0 (15 - 50)	50.0 (27 - 90)	45.0 (20 - 80)
Smoke-free (years)	–	8.0 (2 - 15)	6.0 (1 - 37)	3.5 (1 - 21)

* Median and range () are indicated for each parameter. FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Table 2

Histological characteristics of bronchial epithelial remodelling *

Study groups	Normal-appearing epithelium	Goblet cell hyperplasia
Never	1. Detected in 100% of tissue specimens	1. Detected in 44.4% of tissue specimens
Smokers without COPD	2. Covered 99.2% (range 69.6–100%) of bronchial mucosa	2. Covered 0.8% (range 0–30.4%) of bronchial mucosa
Former	1. Detected in 100% of tissue specimens	1. Detected in 53.8% of tissue specimens
Smokers without COPD	2. Covered 94.3% (range 41.9–100%) of bronchial mucosa	2. Covered 5.7% (range 0 – 58.1%) of bronchial mucosa
COPD I-II	1. Detected in 95.5% of tissue specimens 2. Covered 48.8% ^{††} (range 5.8-100%) of bronchial mucosa	1. Detected in 72.7% [†] of tissue specimens 2. Covered 33.3% ^{††} (range 0-94.1%) of bronchial mucosa
COPD III-IV	1. Detected in 47.8% ^{††} of tissue specimens 2. Covered 1.6% ^{††} (range 0-94.3%) of bronchial mucosa	1. Detected in 91.3% ^{††} of tissue specimens 2. Covered 46.1% ^{††} (range 0-100%) of bronchial mucosa

* Median and range () are indicated for determination of the extent of goblet cell hyperplasia.

[†] $p < 0.05$ compared to never smokers without COPD.

^{††} $p < 0.01$ compared to never smokers without COPD.