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Decreased phosphatase PTEN amplifies PI3K signaling and enhances pro-inflammatory cytokine release in COPD

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Author’s contributions

SY conducted assay, carried out the data analysis and drafted the manuscript. JB, CV, PF and LD were involved in sample preparation and participated in the design of the original study. KI contributed to the data analysis, design of the study and the manuscript preparation. PB participated in the design of the study, and contributed
substantially to preparation of manuscript.

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ABSTRACT

The phosphatidylinositol 3-kinase (PI3K) pathway is activated in chronic obstructive pulmonary disease (COPD), but the regulatory mechanisms for this pathway are yet to be elucidated. The aim of this study was to determine the expression and role of phosphatase and tensin homolog deleted from chromosome 10 (PTEN), a negative regulator of the PI3K pathway, in COPD. PTEN protein expression was measured in the peripheral lung of COPD patients compared to smoking and non-smoking controls. The direct influence of cigarette smoke extract (CSE) on PTEN expression was assessed using primary lung epithelial cells and a cell line (BEAS-2B) in the presence or absence of L-buthionine-sulfoximine (BSO) to deplete intracellular glutathione. The impact of PTEN knock-down by RNA interference on cytokine production was also examined. In peripheral lung, PTEN protein was significantly decreased in patients with COPD compared to the subjects without COPD (p < 0.001), and positively correlated with the severity of air-flow obstruction (FEV₁ % predicted; r = 0.50; p = 0.0012). Conversely, phosphorylated Akt, as a marker of PI3K activation, showed a negative correlation with PTEN protein levels (r = -0.41; p = 0.0042). Both in primary bronchial epithelial cells and BEAS-2B cells, CSE decreased PTEN protein, which was reversed by N-acetyl cysteine treatment. PTEN knock-down potentiated Akt phosphorylation and enhanced production of pro-inflammatory cytokines, such as IL-6, CXCL8, CCL2 and CCL5. In conclusion, oxidative stress reduces PTEN protein levels, which may result in increased PI3K signaling and amplification of inflammation in COPD.

Key words: COPD, PTEN, PI3K, IL-6, oxidative stress
Introduction

Chronic obstructive pulmonary disease (COPD) is associated with amplified inflammatory responses, predominantly in small airways and lung parenchyma (16, 18). Long term inhalation of noxious gases such as cigarette smoke, are the main causal mechanism for this persistent inflammation (31, 36, 38, 43, 48). Although a multiplicity of cells and mediators are involved in the pathophysiology of COPD (7), many reports have suggested the importance of phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt, both of which are strongly up-regulated by the oxidants (5, 20, 28). Recently, we have shown that the PI3K is significantly activated in peripheral blood mononuclear cells, and associated with the COPD disease progression, partly through corticosteroid resistance (44). These results suggest that the oxidant induced enhancement of PI3K pathway has been altered in patients with COPD, and may contribute to its persistent inflammation. In addition, PI3K signaling (determined by Akt phosphorylation) was reported to be more activated in airway epithelial cells collected from COPD subjects when compared those from healthy subjects (15). However, the regulatory mechanisms for prolonged activation of PI3K and its downstream signaling pathway have not yet been determined.

Phosphatase and Tensin Homolog Deleted from Chromosome 10 (PTEN) is a well-described negative regulator of PI3K, which converts phosphatidylinositol-3,4,5-phosphate (PIP3) to phosphatidylinositol-4,5-phosphate (PIP2), leading to the inactivation of downstream target Akt and/or phosphoinositide-dependent kinase (PDK)1 (41). PTEN was originally discovered as a tumor suppressor gene that encodes a phosphatase involved in inactivating a growth and differentiation (27, 42), and was reported to be frequently mutated or deleted in the epithelium of smokers and in lung cancer (22). However, only limited
research has been conducted to elucidate the role of PTEN in the pathogenesis of COPD, despite the fact that COPD is another important disease associated with cigarette smoking. Single nucleotide polymorphism analysis has demonstrated that PTEN polymorphism as an important risk factor for COPD (19). In addition, in vitro data have demonstrated that cigarette smoke extract (CSE) reduces PTEN protein levels (40) (Reference updated), and that oxidative stress impairs its activity (4). Therefore, the present study was designed to clarify whether PTEN in lung is down-regulated in patients with COPD. In addition, we also investigated whether reduced PTEN protein by oxidative stress in bronchial epithelial cells was linked to the secretion of pro-inflammatory cytokines that are relevant to the pathophysiology of COPD.
Methods and Materials

Reagents

Commercially available reagents were obtained as follows: RPMI medium 1640 (RPMI 1640) (#11875), silencer® select negative control No.1 siRNA (#4390844) as transfection negative control for knock down (KD) experiments, Lipofectamin RNAiMAX (#13778), polymerase chain reaction (PCR) primers for PTEN (Hs00829813), GNB2L1 (Hs00272002), interleukin (IL)-6 (Hs00174131), CXCL8 (Hs00174103), MMP-9 (HSs00234579), Muc5ac (Hs00873651), Muc5b (Hs00861595), TGF-β (Hs00998133) were from Life Technologies (Carlsbad, CA); fetal bovine serum (FBS), s-nitroso-n-acetyl-dl-penicillamine (N3398), complete protease inhibitor cocktail (11836153001), thiazolyl blue tetrazololium bromide for MTT assay (M2003), L-buthionine-sulfoximine (BSO), MG-132 (Z-Leu-Leu-Leu-CHO) (C2211), hydrogen peroxide (H₂O₂) (H1009), N-acetyl cysteine (A9165) and dexamethasone (D1756) from Sigma-Aldrich Co.LLC (St Louis, MA); rabbit-derived anti-PTEN antibody (ab154812), anti-β-actin antibody (ab6276) from Abcam plc. (Cambridge, UK); anti-phosphorylated-Akt (Ser47) (p-Akt) antibody (#9271), anti-total Akt antibody (#4691), and PTEN short interference (si) RNA (#6538) from Cell Signaling Technology (Danvers, MA, USA); anti-Nrf2 antibody (sc-13032) from Santa Cruz Biotechnology (Santa cruz, CA); ALLN (N-acetyl-Leu-Leu-Norleu-al) (#208750) from EMD Millipore Corporation (Billerica, MA); recombinant human IL-1β was R&D Systems (Minneapolis, MN); and goat-derived peroxidase-conjugated anti-mouse (P0447) or anti-rabbit (P0448) secondary antibodies from Dako (Cambridge shire, UK).
Peripheral Lung Tissue

COPD patients were categorized according to GOLD stage (35). Peripheral lung tissues from subjects with normal lung function (non-smokers = NS, 8 subjects), smokers without COPD (SM, 9 subjects) and 26 patients with mild- to very severe-COPD (Stage 1, 9 subjects; stage 2, 8 subjects; Stage 3, 3 subjects; and Stage 4, 6 subjects) were obtained using a tissue bank linked to an established patient registry from the patients who have made a decision to proceed with lung resection for the treatment of a lung tumor (12), and protein extracts were prepared using RIPA buffer (Sigma-Aldrich: 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate-polyacrylamide (SDS), and 50 mM Tris-HCl, pH 8.0.) completed with protease inhibitor as previously described (21). We analyzed PTEN protein expression in a blinded manner by Western blotting.

Immunoprecipitation and PTEN activity assay

The protein extract of peripheral lung tissues were immunoprecipitated using an anti-PTEN antibody, and its phosphatase activity measured by a p-nitrophenyl phosphatase (pNPP) protein phosphatase assay kit (AS-71105: AnaSpec, Inc. Fremont, CA) following the manufacturer’s instructions. Briefly, 200μg/200μl of protein extracts were reacted on a shaker platform with 8μl of rabbit-derived anti-PTEN antibody (#9188: Cell Signaling Technology, Danvers, MA) overnight. Then 30μl of Protein A magnetic beads (#88845; Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL) were added, and incubated on the shaker at 4 °C for 4 hours. After washing four times with 100 mM Tris-HCl (pH 8.0), the PTEN combined beads were dissolved in a total 100μl of PTEN reaction buffer (100 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol) (30) complemented with pNPP solution,
and incubated at 37 °C for the appropriate duration. Then the supernatant was transferred to a 96-well microplate, and optical densities measured at a wavelength of 405nm. The results of pNPP assay were calculated by subtracting the OD<sub>405</sub> value of negative control from OD<sub>405</sub> of each sample. In addition, the PTEN proteins were eluted from the beads by boiling, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and detected by Western blotting with mouse-derived anti-PTEN antibody (sc-7974: Santa Cruz Biotechnology, Santa Cruz, CA). Finally, PTEN activity was normalized by PTEN protein level (PTEN activity / protein).

**Cell Culture and Stimulation**

Human primary bronchial epithelial cells were cultured as monolayers in LHC-9 media (Invitrogen, Paisley, UK) on collagen (1% w/v) coated plates. Cells were extracted from lung tissue from patients undergoing lung resection surgery at the Royal Brompton Hospital. All subjects gave informed written consent and the study was approved by the NRES London-Chelsea Research Ethics committee (study number 09/H0801/85). The BEAS-2B cell line (SV40-immortalized human airway bronchial epithelial cell line) were purchased from the American Culture of Tissue Collection, and were maintained in complete growth medium (RPMI 1640 supplemented with heat-inactivated 10% FBS and 1% L-glutamine) at 37 °C / 5% CO<sub>2</sub>. Before use, cells were starved for 24 hours in minimum medium (RPMI 1640 supplemented with 1% FBS and 1% L-glutamine). N-acetyl cysteine (10 mM) was also given 10 min before CSE treatment.

**Preparation of CSE**

One full-strength Marlboro cigarette with filter removed (Phillip Morris, London,
UK) was bubbled into 10 ml of minimum medium, at a rate of one cigarette per 1.5 minutes. CSE was then passed through a 0.2μm filter to sterilize and remove particulate matter and was used immediately. The optical density was measured at 320λ wavelength, and the solution was diluted to be OD=0.85 (this is original stock as 100%). The stock CSE was thereafter diluted with culture media to appropriate percentages of CSE solution.

**RNA interference**

BEAS-2B cells were transfected with PTEN short interference (si) RNA (100 nM) or random oligonucleotide control (RO, 100 nM) for 48 hour using Lipofectamin RNAiMAX, according to the manufacture’s instructions.

**Western blotting**

After stimulation, whole cell extracts were prepared using RIPA buffer (19), separated by SDS-PAGE, transferred to nitrocellulose membrane, and then incubated with anti-PTEN antibody (1:1000 dilutions), anti-phosphorylated-Akt (p-Akt) antibody (1:500 dilutions), anti-total-Akt antibody (1:1000 dilutions) or anti-Nrf2 antibody (1:1000, dilutions) overnight. To standardize the expression of each protein, the membranes were re-probed with anti-β-actin antibody (1:200,000 dilutions). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (1:3000 dilutions, each). The bound antibodies were visualized by chemiluminescence (ECL plus; GE Healthcare, Buckingham, UK).

**Reverse Transcriptase Quantitative PCR (RT-qPCR)**

Total cellular RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) and
cDNA was prepared by using Multiscribe reverse transcriptase (Applied Biosystems, Warrington, UK). The RT-qPCR analysis of PTEN, IL-6, CXCL8, MUC5AC, MUC5B, MMP9, TGFβ and GNB2L1 as a housekeeping gene, were performed using Taqman primers and probe set from Applied Biosystems in a Corbett Rotor-Gene 3000 (Corbett Research Sortlake, Sydney, Australia).

**Cytokine Enzyme-Linked Immunosorbent Assay (ELISA) assay**

Cytokine concentrations in the cell supernatant was assessed by human cytokine array panel A (ARY005: R&D systems, Minneapolis, MN), and concentrations of IL-6, CXCL8, CCL2 and CCL5 were determined by a human sandwich ELISA-kit (R&D Systems) according to the manufacturer’s instructions.

**Statistical Analysis**

Data from clinical samples were expressed as mean values ± SD. For the analysis of PTEN, statistical significance was assessed by Mann-Whitney U test for single comparisons, or by using non-parametric Kruskal–Wallis test with appropriate post hoc analysis (Dunnett’s test) to exclude possible interaction between various variables within subgroups (Statcel 2, OMS publishing Inc., Saitama, Japan). The analysis of correlation between each factors were performed by Spearman’s correlation coefficient rank test. The in vitro data using BEAS-2B cells were expressed as mean values ± SEM. Data were analyzed by one way ANOVA followed by Turkey’s or Sheffe’s F-test to adjust for multiple comparisons. For N-acetyl cysteine treatment study, data were analyzed by one way repeated ANOVA followed by Dunnett’s multiple comparison test. If the parametric analysis was not applicable, Friedman test followed by Dunnett’s multiple comparison was performed.
An unpaired two-tailed Student’s t-test was used for single comparisons. All reported
$P$ values are two-sided, and $P$ values of less than 0.05 were considered statistically
significant.
Results

PTEN expression in peripheral lung and epithelial cells from COPD patients

The characteristics of the subjects are shown in Table 1. We first confirmed the activation status of the PI3K pathway by phosphorylation of its downstream kinase Akt by Western blotting. The phosphorylated fraction of Akt normalized to total Akt protein levels (p-Akt/total-Akt ratio) was significantly increased in COPD lung and was negatively correlated with the FEV\textsubscript{1} percent predicted (%FEV\textsubscript{1}) \((r = -0.41; p < 0.01; \text{Figure 1A})\), as we have previously reported (44). In these samples, the PTEN protein levels were significantly decreased in all patients with COPD, compared with those of the non-smoking subjects with normal lung function (NS). The levels of PTEN protein in smokers without COPD (SM) was also significantly reduced compared with NS \((p<0.05; \text{Figure 1B})\). In addition, there was a significant positive correlation between the PTEN protein levels and airway obstruction measured by FEV\textsubscript{1}/FVC ratio \((r = 0.65, p < 0.001)\) or the severity of air-flow limitation by %FEV\textsubscript{1} \((r = 0.50; p < 0.01; \text{Figure 1C})\), indicating that the PTEN levels were decreased significantly as COPD progresses (Figure 1D). Furthermore, the level of PTEN protein was negatively correlated with phosphorylated fraction of Akt \((p-Akt/total-Akt) \((r = -0.58; p < 0.01; \text{Figure 1E})\); which suggested the importance of PTEN in regulating the Akt phosphorylation \textit{in vivo}. Despite the long smoking history of COPD patients, there was no apparent relationships between the PTEN protein levels and smoking status \((r = -0.22; p = 0.16)\) or with age \((r = -0.12; p = 0.42)\) or gender \((p = 0.81)\). Interestingly, there was a negative correlation between PTEN protein levels and the level of malondialdehyde (MDA), an oxidative stress marker \((r = -0.75, p<0.01, \text{Figure 1F})\). MDA was also correlated with p-Akt/total-Akt ratio \((r = 0.36, p<0.05) (\text{Figure 1G})\). In addition, we also determined mRNA levels of
PTEN in COPD lung samples. There was a trend of the reduction of mRNA of PTEN normalized to a housekeeping gene GNB2L1, but not statistically significant (NS: 0.25 ± 0.063, SM: 0.22 ± 0.056, GOLD I/II: 0.22 ± 0.048, GOLD III/IV: 0.11 ± 0.021).

As the peripheral lung contains many different type of cells, we examined PTEN protein levels in primary human bronchial epithelial cells isolated from subjects with or without COPD (Table 2) in the presence or absence of oxidant exposure (CSE). As shown in Figure 1H, COPD patients showed the decreased protein levels of PTEN at baseline, which was further reduced in the presence of CSE in a dose dependent manner (Figure 1H); therefore PTEN protein expression was reduced in bronchial epithelial cells and the cells seemed to be an important target of exogenous oxidative stress with resultant PTEN reduction.

PTEN activity in peripheral lung from COPD patients

We also examined the levels of immunopurified (IP) PTEN activity (Figure 2A) using the pNPP assay. The reliability of pNPP assay was first confirmed using samples from bronchial epithelial BEAS-2B cell line in vitro. As shown in Figure 2B, IP-PTEN activity collected from BEAS2B cells treated with hydrogen peroxide (H₂O₂; 2 mM for 10 min at 37 °C) was significantly reduced. The activity of IP-PTEN was also reduced by treatment of S-nitrosothiol (SNO; 1 mM for 10 min at 25 °C) (Figure 1C). Thus in BEAS2B cells, oxidative stress potentially reduced PTEN activity, as previously reported (23, 24, 52). In this assay system, we examined PTEN activity immunopurified from peripheral lung samples available. As the assay was underpowered, we did not find any difference of the IP-PTEN activities between COPD patients and non-COPD subjects. In addition, there was no
significant correlation between the IP-PTEN activities determined in this condition
and the FEV₁/FVC (p = 0.63) nor %FEV₁ (p = 0.66, Figure 2D).

CSE-induced PTEN reduction in bronchial epithelial cells

On the basis of data from clinical samples, we explored the molecular mechanisms of
PTEN reduction using BEAS-2B cells in vitro. When BEAS-2B cells are
pre-incubated with L-buthionine-sulfoximine (BSO), an irreversible inhibitor of
γ-glutamylcysteine synthetase which depletes the intracellular glutathione (13), the
PTEN protein levels were significantly reduced after 24 hours exposure with as low
as 3% CSE in the presence of 100 μM BSO (Figure 3A). Under these conditions
there was no reduction in cell viability (relative cell viability vs. non-treated cells:
0.90 ± 0.04, not statistically significant)data not shown). Conversely, Akt was
significantly phosphorylated (Figure 3A), as observed in clinical samples. The
reduction of PTEN and an increase in Akt phosphorylation by 3% of CSE at 24 h
were significantly inhibited by pretreatment of an anti-oxidant agent, N-acetyl
cysteine (10 mM) (Figure 3B), suggesting the reduction of PTEN was oxidative
stress-dependent. In addition, the mechanisms for Akt phosphorylation seemed to
differ between early- and late-phase after CSE exposure; although the acute Akt
phosphorylation was transient and did not accompany the suppression of PTEN
protein level (Figure 3C), the Akt phosphorylation at later time points was associated
with reduced PTEN expression (Figure 3D). Thus, PTEN reduction contributes to the
prolonged phosphorylation of Akt even 24 hours after CSE exposure (Figure 3D).

Protein degradation was not responsible for the reduction in PTEN protein
levels as the proteasome inhibitors MG-132 (Z-Leu-Leu-Leu-CHO) and ALLN
(N-acetyl-Leu-Leu-Norleu-al) did not reverse the reduction in PTEN protein levels after the oxidative stress (Figure 4A). By contrast, the mRNA of PTEN decreased significantly but partially in CSE exposure with BSO pretreatment group (Figure 4B), which occurred as early as 6 hours after CSE exposure in advance of the PTEN protein reduction (Figure 4C). Therefore, reduced PTEN gene transcription by oxidative stress is partially involved in reducing the PTEN protein levels. As mentioned above, we observed reduction of PTEN mRNA in peripheral lung from GOLD stage 3+4 although it was not statistically significant.

**PTEN-knock down (KD) caused Akt phosphorylation and enhanced cytokine production**

To investigate the functional consequence of PTEN reduction, PTEN was knocked down in BEAS-2B cells and several cytokine levels were evaluated. After 48 h incubation with PTEN siRNA (100 nM), we could effectively knock-down both the mRNA (Figure 4A) and the protein levels of PTEN (Figure 4B). The knock-down of PTEN was accompanied with the significant phosphorylation of Akt (p-Akt/total-Akt) (Figure 4B lower panel), and enhanced the secretion of various cytokines, such as IL-6, CXCL8, CXCL10, CCL2 and CCL5 (Figure 4C) in the cytokine array assay, all of which are known to be associated with the pro-inflammatory response in the pathogenesis of COPD (6). As the cytokine array assay is semi-quantitative, ELISA, a more quantitative method was then used, and we confirmed up-regulation of IL-6 (Figure 4F), CXCL8 (Figure 4G), CCL5 (Figure 4H) and CCL2 (Figure 4I) after PTEN-KD.

As well as basal cytokine production, IL-1β-induced CXCL8 secretion was also significantly potentiated in PTEN-KD cells and the dose-response curve shifted
leftward ($EC_{50} 0.093 \pm 0.011$ vs. $0.031 \pm 0.014$, $p < 0.05$) (Figure 4J).

The increase in IL-6 and CXCL8 was also confirmed at transcription level (mRNA) by RT-PCR (Figure 4K and L, respectively). In addition, we also confirmed that MMP-9 and TGF-β gene expression was elevated in PTEN knock down cells, but the gene expression of MUC5AC and MUC5B were not affected (Figure 4M, N, O, P).
In the current study, we have shown for the first time that the PTEN protein levels were significantly decreased in the peripheral lung of patients with COPD. PTEN protein levels were positively correlated with the severity of air-flow obstruction, and showed a strong negative correlation with the Akt phosphorylation, indicating activation of the PI3K signaling pathway. This is consistent with the modulatory role of PTEN on PI3K signaling (41).

The PI3K signaling pathway is an important signal cascade that may be activated by oxidative stress (20), and its prolonged/elevated and inappropriate activation is associated with various pulmonary diseases, such as lung cancer (2, 3, 51), interstitial lung disease (33, 34, 47, 49), and COPD (32, 44, 50). Previously, reports in lung cancer demonstrated a reduction in PTEN as consequence of smoking, but these reports did not differentiate COPD from smokers without airway obstruction. Therefore, it might be possible that PTEN reduction is specific to COPD, even though cigarette smoking is the major risk factor involved in the development of both the COPD and lung cancer (17). The peripheral samples we used were obtained from the tissue bank which collected lung samples from the patients who have made a decision to proceed with lung resection for the treatment of a lung tumor. Therefore, all subjects suffered from low grade or moderate different type of cancer (although non-cancerous tissue was used for analysis). This also suggested that the reduction of PTEN is more associated to COPD rather than cancer status. Indeed, reduced PTEN may contribute to the greatly increased risk of lung cancer in COPD patients (1).

Many reports have elucidated the inhibitory effect of the oxidative stress on the PTEN phosphatase activity (23–26, 39, 52), especially via redox regulation of
reactive cysteine (Cys124) at the catalytic site of the enzyme. Contrary to our expectations, we could not detect any reductions of IP-PTEN phosphatase activities (normalized by its protein level) in samples from the patients with COPD. In this study, we could collect IP-PTEN protein samples only from lung tissue from limited subjects, therefore, the assay was totally underpowered and also, a specific PTEN activity assay system is not available, nor are antibodies targeting oxidized PTEN currently available. This potentially limits the measurement of PTEN activity under conditions of oxidative stress. Therefore, it is inconclusive whether the PTEN activity was reduced in COPD or not.

In our BEAS-2B cell model, higher concentrations of CSE (20%) effectively down-regulated the expression of PTEN protein (PTEN/β-actin relative ratio vs non-treated control: 0.077 ± 0.028, 92.3% reduction data not shown). This was compatible with a previous report that oxidative stress suppresses PTEN expression in human airway epithelial cells (40). However, as the cell toxicity by such a high concentration of CSE was substantial, we could not rule out the possibility of PTEN degradation as a result of cell damage and death. These effects with higher concentration of CSE might be associated with the acute toxicity of cigarette smoke exposure (33), however it is difficult to use this model for exploring the pathogenesis of COPD, which is more chronic reaction to lower concentrations of cigarette smoke. Therefore, we next examined PTEN protein expression in the BSO pretreatment model, which depletes intracellular glutathione storage and may mimic the reduction in glutathione seen in COPD cells. BSO treatment enhances sensitivity of cells to oxidative stress, and therefore, we only need low concentrations of CSE to avoid any reduction of cell viability seen in high concentrations of CSE. Glutathione concentrations in sputum (46) or muscle (14) are reduced in patients with COPD, and
cigarette smoke itself plays a critical role in depleting available glutathione stores in
airway epithelial cells (45). Therefore, the combination of CSE exposure with BSO
pretreatment may be a more appropriate model of what occurs in vivo in COPD
patients. In fact, BSO augmented the effect of CSE in this model, and as low as 3%
CSE decreased the protein level of PTEN in a time-dependent manner, without any
significant cell injury. This partially explains the different sensitivity against
cigarette smoke between the smokers without COPD and patients with COPD as
demonstrated by the experiments with primary bronchial epithelial cells from COPD
patients (Figure 1H). The replenishment of intracellular glutathione might be more
effective than the oral intake of anti-oxidants (37). In addition, the PTEN reduction
by CSE was reversed by N-acetyl cysteine treatment, suggesting the involvement of
oxidative stress on PTEN reduction (Figure 3B). In fact, we also demonstrated a
good negative correlation between the levels of PTEN protein and MDA, a product
of lipid oxidation by reactive oxygen species, in peripheral lung tissue (Figure 1F).

Our data show that the PI3K pathway appears to be activated by different
mechanisms according to duration of CSE exposure (Figure 3C-3D). In the early
phase after CSE exposure, Akt was phosphorylated directly by the oxidative stress,
without accompanying the PTEN suppression. At later time point, there was a
gradual reduction in PTEN, causing prolonged phosphorylation of Akt, even after the
disappearance of CSE. These different responses with time suggest that the PTEN is
more important for the late-phase reaction to CSE. Also, this might explain why the
chronic inflammation progress even after the cessation of smoking (10), and why
some patients may be more prone to acute exacerbations (29).

We also examined the PTEN-KD model using siRNA to reduce PTEN,
which resulted in increased p-Akt even under basal conditions, and also enhanced the
secretion of several pro-inflammatory cytokines, including IL-6, CXCL8, CXCL10
and CCL5, all of which are increased in the sputum of COPD patients (9, 11). This
effect on basal secretion implies a potent basal inhibitory effect of PTEN on normal
PI3K signaling. In addition, PTEN-KD augmented IL-1β-induced CXCL8 secretion,
and shifted the dose-response curve of IL-1β stimulation to the left. These results
suggested that the PTEN-KD cells might be used as the reasonable in vitro model of
COPD, with which we can imitate and reproduce the cell responses beyond just CSE
exposure. Furthermore, we also confirmed that PTEN regulated gene expression of
MMP9 and TGF-β as well as IL-6 and CXCL8, but did not for MUC5AC and
MUC5B, all were involved in pathogenesis of COPD. Thus, PTEN controlled
expression of specific cytokine or biological factors. Further broad and systematic
analysis will be required to clarify the impact of PTEN reduction in all aspect of
airway inflammation in future studies.

In conclusion, we have shown that the oxidative stress inhibits the protein
levels of PTEN in patients with COPD, resulting in the persistent activation of the
PI3K/Akt pathway and resultant pro-inflammatory mediator release. This may
partially explain why COPD progresses even after the cessation of smoking, and why
some patients are prone to frequent exacerbations. In addition activation of PI3K
signaling as a result of decreased PTEN expression may also be important in
corticosteroid resistance and accelerated aging as well as the increased risk of lung
cancer in COPD (8). Enhancement of the anti-inflammatory PTEN function might be
a possible future therapeutic target in preventing COPD progression.
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Disclosure

PJB has served on Scientific Advisory Boards of AstraZeneca, Boehringer-Ingelheim, Chiesi, GlaxoSmithKline, Glenmark, Johnson & Johnson, Napp, Novartis, Takeda, Pfizer, Prosonix, RespiVert, Teva and Zambon and has received research funding from AstraZeneca, Boehringer-Ingelheim, Chiesi, Novartis and Takeda.; KI is currently an employee of Pulmocide Ltd and has honorary contract with Imperial College.
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Figure Legends

Figure 1. PTEN expression in peripheral lung and bronchial epithelial cells from COPD. Whole tissue extracts were prepared from peripheral lung tissue of non-smokers (NS; n=8), smokers without COPD (SM; n=9), patients with COPD stage 1 (C1; n=9), COPD stage 2 (C2; n=8), COPD stage 3 (C3; n=3) and COPD stage 4 (C4; n=6) in lysis buffer, and phosphorylated-Akt (p-Akt), total-Akt, PTEN and β-actin protein levels were determined by Western blotting. (A) Correlation between the forced expiratory volume in 1 second percent predicted (%FEV₁) and phosphorylated fraction of Akt (p-Akt/total-Akt). (B) PTEN protein level in lung sample from healthy non-smoker subjects (NS) and smokers without COPD (SM) and COPD patients (C1-4). (C) Correlation of PTEN protein levels and the %FEV₁. (D) Representative Western blot image and PTEN protein levels of healthy subjects (NS, SM) and patients with COPD of each stage C1-4. (E) Correlation of PTEN protein levels and p-Akt/total-Akt. Correlation of malondialdehyde (MDA) with PTEN protein levels (F) and p-Akt/total-Akt (G). (H) Primary bronchial epithelial cells were isolated from the healthy subjects (HS; n=4) or COPD patients (n=4), and PTEN protein levels in the presence or absence of cigarette smoke extract (CSE) were examined by Western blotting. The effect of different concentrations of CSE on PTEN protein in BEAS-2B cells incubated for 24h were shown. * p < 0.05, ** p < 0.01 compared with the values of NS, § p < 0.05, §§ p < 0.01 compared with the each CSE 0% group, †p < 0.05 between the two groups.

Figure 2. PTEN activity in BEAS2B cells and peripheral lung from COPD.
(A) Protein extracts of BEAS-2B cells were immunoprecipitated for PTEN and detected by WB. WCE: whole cell extracts. (B) BEAS-2B cells were treated with or
without 2 mM hydrogen peroxide (H$_2$O$_2$) for 24 h, and PTEN was immunopurified and IP-PTEN activity measured. (C) IP-PTEN was directly treated with 1 mM S-nitrosothiol (SNO), and its activity was measured. (D) Correlation of PTEN activity levels and the %FEV$_1$.

**Figure 3. The effect of CSE on PTEN and PI3K signaling after short- and long-term exposure in BEAS-2B cells.**

(A) After 16 h pretreatment with or without 100 μM L-buthionine-sulfoximine (BSO), BEAS-2B cells were cultured in the presence or absence of the 3% CSE for 24 hours, and then PTEN protein expression (corrected with β-actin) and Akt phosphorylation (corrected with total Akt) were determined by Western blotting (n=3). (B) Effect of N-acetyl cysteine (10 mM, 30 min before CSE stimulation) on CSE (3%) induced reduction of PTEN and elevation of Akt phosphorylation (n=5). (C) Effect of 3% CSE on PTEN (upper) or p-Akt/total-Akt (lower) up to 60 min in the presence or absence of 100 μM BSO. (D) Time-dependent effect of 3% CSE on PTEN (upper) and p-Akt/total-Akt (lower) at 6, 12, 18 and 24 hours after stimulation in the presence or absence of BSO pretreatment. All values are mean values ± SEM of at least three experiments. * $p < 0.05$, ** $p < 0.01$, compared with the values of non-treatment group, † $p < 0.05$, †† $p < 0.01$ between the two groups.

**Figure 4. PTEN reduction by CSE was mediated partially through the mRNA suppression.** (A) BEAS-2B cells were treated with or without 3% CSE and 100 μM BSO co-stimulation in the presence or absence of proteasome inhibitor, MG-132 or ALLN. Then PTEN protein levels were assayed by WB. The Nrf2 was used as the standard protein affected by proteasome inhibitors. (B) BEAS-2B cells were
stimulated with or without 3% CSE in the presence or absence of 16 hours pretreatment of 100 μM BSO. After 24 hour, PTEN mRNA level was examined by Reverse Transcriptase Quantitative Polymerase chain reaction (RT-qPCR). (C) With 3% CSE in the presence of 100 μM BSO pretreatment, PTEN mRNA levels were assayed at each time points by RT-qPCR. All values are mean values ± SEM of at least three separate experiments. * p < 0.05, ** p < 0.01, compared with the values of non-treatment group.

Figure 5. Effect of PTEN-knock down (KD) on Akt phosphorylation and cytokine production. BEAS-2B cells were incubated with random oligonucleotide (RO; 100 nM) or short interference (si) RNA against PTEN (100 nM) for 48 hours. The levels of PTEN were determined by RT-qPCR (A) and western blotting (B). The ratio of p-Akt/total-Akt as well as PTEN/β-actin were also determined (n=3). Effects of PTEN knock-down on cytokine production determined by cytokine array (C), or by ELISA for IL-6 (F), CXCL8 (G), CCL5 (H) and CCL2 (I). (J) Effect of stimulation with IL-1β (0, 0.01, 0.1 and 1 ng/mL) on CXCL8 release for 24 hours in PTEN-KD cells. An impact of PTEN KD on gene expression IL-6 (K), CXCL8 (L), MMP-9 (M), TGF-β (N), MUC5AC (O) and MUC5B (P), all which were corrected with the gene expression of a house keeping gene GNB2L1 (n=3). All values are mean values ± SEM of three to six separate experiments. ** p < 0.01, compared with the values of RO-treated group; † † p < 0.01, compared with the values of non-treatment RO group; ‡ ‡ p < 0.05, ‡ ‡ p < 0.01, compared with the value of non-treatment PTEN-KD group; §§ p < 0.01, between the RO group and PTEN-KD group.
Table 1. The characteristics of study subjects for peripheral lung tissues.

<table>
<thead>
<tr>
<th></th>
<th>Non-smokers</th>
<th>Smokers without COPD</th>
<th>COPD1</th>
<th>COPD2</th>
<th>COPD3</th>
<th>COPD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (M/F)</td>
<td>8 (4/4)</td>
<td>9 (3/6)</td>
<td>9 (5/4)</td>
<td>8 (3/5)</td>
<td>3 (2/1)</td>
<td>6 (3/3)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.6 ± 21.2</td>
<td>64.7 ± 12.7</td>
<td>69.2 ± 6.5</td>
<td>59.5 ± 7.2</td>
<td>63.3 ± 10.2</td>
<td>57.8 ± 4.4</td>
</tr>
<tr>
<td>Pack year</td>
<td>N/A</td>
<td>56.1 ± 34.1</td>
<td>53.0 ± 27.5</td>
<td>57.0 ± 37.8</td>
<td>46.0 ± 12.3</td>
<td>41.0 ± 16.7</td>
</tr>
<tr>
<td>FEV₁ (l)</td>
<td>3.02 ± 0.92</td>
<td>2.53 ± 0.62</td>
<td>2.48 ± 0.62</td>
<td>1.72 ± 0.41##</td>
<td>1.56 ± 0.55##</td>
<td>0.56 ± 0.14##</td>
</tr>
<tr>
<td>%FEV₁ (%)</td>
<td>99.3 ± 18.2</td>
<td>97.0 ± 16.4</td>
<td>90.7 ± 7.5</td>
<td>60.3 ± 7.7##</td>
<td>46.9 ± 1.5##</td>
<td>18.1 ± 3.9##</td>
</tr>
<tr>
<td>FEV₁ / FVC ratio(%)</td>
<td>81.8 ± 3.8</td>
<td>73.6 ± 2.1#</td>
<td>62.6 ± 5.3##</td>
<td>61.6 ± 9.0##</td>
<td>54.5 ± 6.9##</td>
<td>27.9 ± 7.5##</td>
</tr>
<tr>
<td>Inhaled CS</td>
<td>0/8</td>
<td>0/9</td>
<td>0/9</td>
<td>2/8</td>
<td>1/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Systemic CS</td>
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<td>1/9</td>
<td>0/9</td>
<td>1/8</td>
<td>0/3</td>
<td>0/6</td>
</tr>
</tbody>
</table>

COPD patients were categorized by GOLD stage (33).

Abbreviations: COPD = chronic obstructive pulmonary disease; pack-year = \{number of cigarettes smoked per day / 20\} (“pack”) × duration of smoking (“year”); FEV₁ = forced expiratory volume in one second; %FEV₁ = FEV₁ % predicted normal; FVC = forced vital capacity; CS: corticosteroids; # \( p < 0.05 \), ## \( p < 0.01 \) vs. Non-Smokers. Data are expressed as mean values ± standard deviation.
Table 2. The characteristics of study subjects for primary epithelial cells.

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
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</thead>
<tbody>
<tr>
<td>Number (M/F)</td>
<td>3/1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>68.75 ± 9.8</td>
</tr>
<tr>
<td>Pack year</td>
<td>82 ± 58.7</td>
</tr>
<tr>
<td>FEV₁ (l)</td>
<td>1.56 ± 3.1</td>
</tr>
<tr>
<td>%FEV₁ (%)</td>
<td>62 ± 18.2</td>
</tr>
<tr>
<td>FEV₁ / FVC ratio (%)</td>
<td>49 ± 14</td>
</tr>
</tbody>
</table>

COPD patients were categorized by GOLD stage (33). Abbreviations: COPD = chronic obstructive pulmonary disease; pack-year = {number of cigarettes smoked per day / 20} (“pack”) × duration of smoking (“year”); FEV₁ = forced expiratory volume in one second; %FEV₁ = FEV₁ % predicted normal; FVC = forced vital capacity. Data are expressed as mean values ± standard deviation.
Figure 3

(A) BSO:100μM - + - +
CSE:3% - - + +
PTEN
p-Akt
Total-Akt
β-actin

PTEN / β-actin

p-Akt / Total-Akt

(B) BSO:100μM
NAC:10μM
CSE:3%
PTEN
p-Akt
Total-Akt
β-actin

PTEN / β-actin

p-Akt / Total-Akt

(C) BSO:100μM
Time(min) 0 10 20 30 60
PTEN
p-Akt
Total-Akt
β-actin

PTEN / β-actin

p-Akt / Total-Akt

(D) BSO:100μM + CSE:3%
Time (hr) 0 6 12 18 24
PTEN
p-Akt
Total-Akt
β-Actin

PTEN / β-actin

p-Akt / Total-Akt