Targeting Phosphoinositide-3-Kinase- δ with Theophylline Reverses Corticosteroid Insensitivity in Chronic Obstructive Pulmonary Disease

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Rationale: Patients with chronic obstructive pulmonary disease (COPD) show a poor response to corticosteroids. This has been linked to a reduction of histone deacetylase-2 as a result of oxidative stress and is reversed by theophylline.

Objectives: To determine the role of phosphoinositide-3-kinase- δ (PI3K- δ) on the development of corticosteroid insensitivity in COPD and under oxidative stress, and as a target for theophylline.

Methods: Corticosteroid sensitivity was determined as the 50% inhibitory concentration of dexamethasone on tumor necrosis factor- α -induced interleukin-8 release in peripheral blood mononuclear cells from patients with COPD ($n = 17$) and compared with that of nonsmoking ($n = 8$) and smoking ($n = 7$) control subjects. The effect of theophylline and a selective PI3K- δ inhibitor (IC87114) on restoration of corticosteroid sensitivity was confirmed in cigarette smoke-exposed mice.

Measurements and Main Results: Peripheral blood mononuclear cells of COPD (50% inhibitory concentration of dexamethasone: 156.8 ± 32.6 nM) were less corticosteroid sensitive than those of nonsmoking (41.2 ± 10.5 nM; $P = 0.018$) and smoking control subjects (47.5 ± 19.6 nM; $P = 0.031$). Corticosteroid insensitivity and reduced histone deacetylase-2 activity after oxidative stress were reversed by a non-selective PI3K inhibitor (LY294002) and low concentrations of theophylline. Theophylline was a potent selective inhibitor of oxidant-activated PI3K- δ , which was up-regulated in peripheral lung tissue of patients with COPD. Furthermore, cells with knock-down of PI3K- δ failed to develop corticosteroid insensitivity with oxidative stress. Both theophylline and IC87114, combined with dexamethasone, inhibited corticosteroid-insensitive lung inflammation in cigarette-smoke-exposed mice *in vivo*.

Conclusions: Inhibition of oxidative stress dependent PI3K- δ activation by a selective inhibitor or theophylline provides a novel approach to reversing corticosteroid insensitivity in COPD.

Keywords: Histone deacetylase 2; dexamethasone; cigarette smoke; oxidative stress; RNA interference

Chronic obstructive pulmonary disease (COPD) is a major and increasing global health problem. The Global Burden of

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Corticosteroid insensitivity in chronic obstructive pulmonary disease (COPD) has been linked to reduced expression and activity of histone deacetylase-2 as a result of oxidative stress.

What This Study Adds to the Field

We have shown that theophylline increases histone deacetylase-2 and corticosteroid sensitivity *in vitro* and in smoking mice *in vivo* by specifically inhibiting oxidant-activated phosphoinositide-3-kinase- δ (PI3K- δ). PI3K- δ is activated in COPD lungs and by oxidative stress and selective inhibitors of PI3K- δ mimic the effects of theophylline in reversing corticosteroid resistance.

Disease studies predict that COPD will rise to become the third most common cause of death in the world by the year 2020 (1). COPD is caused by long-term inhalation of noxious gases and particles, such as cigarette smoke. COPD is a chronic inflammatory disorder, which is orchestrated by various inflammatory cytokines, chemokines, and mediators (2). However, in contrast to asthma, corticosteroids provide little clinical benefit and do not reduce the progression or the mortality of COPD (3, 4). This may reflect the fact that the chronic lung inflammation in COPD is not suppressed by corticosteroids, even at high doses (5). The molecular mechanism of this corticosteroid insensitivity in COPD involves a reduction in the activity and expression of the critical nuclear enzyme histone deacetylase-2 (HDAC2), causing hyperacetylation of glucocorticoid receptors, which prevents steroids from switching off activated inflammatory genes (3). We have also reported that low concentrations of theophylline restore HDAC2 activity reduced in COPD cells and can thus reverse corticosteroid resistance (6).

Theophylline is an old drug that was originally used as a bronchodilator but has more recently been shown to be antiinflammatory and to enhance corticosteroid actions at lower concentrations (7, 8). However, the molecular mechanisms behind these effects of theophylline are still not well understood. Theophylline directly inhibits phosphodiesterases (PDEs) and antagonizes adenosine receptors at relatively high concentrations and these actions account for all of its known side effects, such as nausea, gastrointestinal disturbance, cardiac arrhythmias, and seizures (9). Theophylline at lower concentrations is also reported to have inhibitory effects on phosphoinositide metabolism and to inhibit phosphoinositide-3-kinase (PI3K) activity (10). PI3Ks generate lipid second messengers

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that control an array of intracellular signaling pathways, which have important roles in inflammation (11). We now show that low concentrations of theophylline restore corticosteroid sensitivity by enhancement of HDAC2 activity through inhibition of the δ -isoform of PI3K and that this is independent from PDE inhibition and adenosine antagonism. We have demonstrated this in circulating cells from patients with COPD *in vitro* and in a tobacco smoke-exposed mouse model *in vivo*. Some of the results of these studies have been previously reported in the form of an abstract (12).

METHODS

Details of the assays are provided in the online supplement.

Materials

LY294002, dexamethasone, and theophylline were purchased from Sigma (Poole, UK) and IC87114 was generously provided by Calistoga Pharmaceuticals, Inc. (Seattle, WA).

Patients and Healthy Volunteers

Peripheral blood mononuclear cells (PBMCs) were obtained from 17 patients with COPD (13), 8 nonsmoking control (NSC) subjects, and 7 smoking control (SC) subjects using Ficoll-Paque (Amersham Bioscience UK Ltd, Little Chalfont, UK) as previously reported (*see* Table E1 in the online supplement) (14).

This study was approved by the local ethics committee of Royal Brompton and Harefield NHS Trust and written informed consent was obtained from each patient or volunteer.

Peripheral Lung Tissue

Peripheral lung tissue from 17 NSC subjects with normal lung function, 15 SC subjects with normal lung function, 10 subjects with stage 1 (mild), 18 with stage 2 (moderate), 6 with stage 3 (severe), and 10 with stage 4 (very severe) COPD, was obtained using a tissue bank linked to an established patient registry (Table E2) (8).

Murine Smoking Model

A/J mice were exposed to 4% cigarette smoke for 30 minutes per day for 11 days using a Tobacco Smoke Inhalation Experiment System for small animals (Model SIS-CS; Shibata Scientific Technology, Tokyo, Japan), as described previously (15). Bronchoalveolar lavage (BAL) fluid was collected to determine the number of alveolar macrophages and neutrophils. This animal study was approved by the Ethics Review Committee for Animal Experimentation of Nihon University.

IL-8 Assay

IL-8 in supernatant was determined using human CXCL8/IL-8 DuoSet ELISA (R&D Systems, Minneapolis, MN).

Detection of pAkt by Western Blotting

Whole-cell extracts were prepared as shown previously, and phosphorylated-pan Akt or total pan Akt was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blotting with antibody (Millipore/Upstate, Watford, UK).

Phosphatidylinositol Trisphosphate Assay

The PI3K activity in whole-cell homogenates or immunoprecipitated PI3K- δ was determined using a phosphoinositide 3,4,5-trisphosphate (PIP₃) Mass ELISA kit (Echelon Biosciences, Salt Lake City, UT).

RNA Interference

PI3K- δ , PI3K- γ , or HDAC2 short interference (si) RNA and scrambled oligonucleotide as a transfection control (Ambion, Huntingdon, UK) were transfected using Nucleofector and AMAXA T-kit (Amaza Biosystems, Cologne, Germany).

Reverse Transcriptase Polymerase Chain Reaction

Gene transcript level of PI3K- α , PI3K- γ , PI3K- δ , and GNB2L1, as a house keeping gene, were quantified by real-time polymerase chain reaction using Taqman primers (Applied Biosystems, Warrington, UK) on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia).

HDAC2 Activity

HDAC2 was immunoprecipitated and the activity was measured by Fluo-Lys HDAC activity assay kit (Biomol International UK, Exeter, UK) as previously reported (12).

Statistical Analysis

Results are expressed as means $\pm$ SEM. Analysis of variance was performed by Kruskal-Wallis analysis and when significant comparisons were made by Mann-Whitney U test using GraphPad Prism (GraphPad Software, San Diego, CA). One-way analysis of variance, Wilcoxon matched-pairs test, Spearman correlation, and Student's *t* test were also used to determine significance when applicable. *P* values less than 0.05 were considered to be significant.

RESULTS

Restoration of Corticosteroid Sensitivity by a PI3K Inhibitor in PBMCs from COPD Cells *In Vitro*

PBMCs were stimulated with tumor necrosis factor (TNF)- α in the presence or absence of dexamethasone (10^{-11} – 10^{-6} M) and supernatant collected for assay of IL-8 by ELISA. TNF- α -stimulated IL-8 production was 499.3 ± 89.5 pg/ml in PBMCs obtained from moderate to severe COPD ($n = 17$), which was greater than from NSC (403.5 ± 53.7 pg/ml; $n = 8$) or from SC (492.2 ± 79.1 pg/ml; $n = 7$), but this difference did not achieve statistical significance (Figure 1A).

Dexamethasone inhibited TNF- α -induced IL-8 production in a concentration-dependent manner, and the concentration-response curves in COPD cells were shifted rightward, indicating reduced steroid sensitivity compared with NSC and SC cells (Figure E1A). The 50% inhibitory concentration of dexamethasone (IC₅₀-Dex) was 156.8 ± 32.6 nM in COPD cells ($n = 17$), which was significantly greater than that in NSC (41.2 ± 10.5 nM; $P = 0.018$; $n = 8$) and SC cells (47.5 ± 19.6 nM; $P = 0.031$; $n = 7$) (Figure 1B). There was a significant correlation between IC₅₀-Dex and FEV₁% predicted in patients with COPD (Spearman $r = -0.65$; $P = 0.0046$) (*see* Figure E1B), suggesting that disease severity was associated with corticosteroid insensitivity (Figure 1C). When EC₅₀-Dex values are calculated, there is still good correlation between EC₅₀-Dex and FEV₁% predicted in patients with COPD (Spearman's $r = -0.65$; $P = 0.0046$).

Although LY294002, a pan-isoform PI3K inhibitor (LY 10^{-6} M; 517.2 ± 114.4 pg/ml; $n = 16$), did not inhibit IL-8 production in PBMCs from patients with COPD (499.3 ± 86.4 pg/ml; $n = 16$) (*see* Figure E1C), LY (10^{-6} M) significantly restored corticosteroid sensitivity in cells from patients with COPD (IC₅₀-Dex: 50.3 ± 8.2 nM with LY vs. 147.7 ± 41.3 nM; $P = 0.0315$; $n = 12$) (Figure 1D).

Activation of PI3K in COPD

As a surrogate marker of PI3K activation, phosphorylation of the downstream target enzyme Akt (pAkt) was determined by Western blotting and normalized to total Akt protein. Basal pAkt level showed a trend (but not significant) to be increased in PBMCs of patients with COPD (pAkt/total Akt ratio: NSC 0.83 ± 0.21 , $n = 8$; SC 0.96 ± 0.50 , $n = 7$; COPD 1.2 ± 0.44 , $n = 8$) (*see* Figure E1D). There was a good correlation between the efficacy of LY in restoring IC₅₀-Dex and the level of pAkt (Spearman $r = 0.81$; $P = 0.0218$) (*see* Figure E1E). Thus, the

Figure 1. Phosphoinositide-3-kinase (PI3K) inhibitor reversed corticosteroid insensitivity seen in peripheral blood mononuclear cells (PBMCs) obtained from chronic obstructive pulmonary disease (COPD). PBMCs were separated from 8 nonsmoking control subjects (NSC), 7 smoking control subjects (SC), and 17 patients with COPD. The cells were stimulated with tumor necrosis factor (TNF)-α (1 ng/ml) with or without serial concentrations of dexamethasone (Dex, 10^{-11} – 10^{-6} M). Supernatants were taken after overnight incubation for interleukin(IL)-8 ELISA. (A) The average value of IL-8 with or without TNF-α in each group is shown; NS = not significant. (B) IC₅₀-Dex values were calculated and plotted individually. (C) The correlation between FEV₁ (% predicted) and IC₅₀-Dex in patients with COPD (n = 17) analyzed by Spearman correlation test. (D) IC₅₀-Dex values on TNF-α-induced IL-8 production were calculated individually in the presence or absence of LY-294002 (LY) in PBMCs obtained from 12 patients with COPD.

patients with higher levels of pAkt responded better to the PI3K inhibitor. In addition, we reanalyzed the effects of LY294002 on restoration of dexamethasone sensitivity pharmacologically. Because we found responder/nonresponder from the results (Figure 1C and Figure E1D), we divided patients with COPD into two groups based on Akt phosphorylation level by cutoff point of 0.05 (pAkt/Akt). We then calculated EC₅₀ (instead of IC₅₀) and also E-max of dexamethasone. As shown in Table 1, LY294002

clearly restored EC₅₀ values in PI3K activated (higher pAkt) samples and did not affect the dexamethasone sensitivity in the low pAkt group. Interestingly, LY294002 did not have any impact on E-max. Thus, there are obvious responders to LY294002 or nonresponders, and we are able to identify the responders based on PI3K activation in the samples (pAkt level).

The ratio of pAkt was also elevated in peripheral lung tissue in COPD compared with those of NSC (Figure 2A) (pAkt/Akt ratio: NSC 0.019 ± 0.0043 , n = 16; SC 0.044 ± 0.010 , n = 13; mild COPD (GOLD stage 1), 0.034 ± 0.082 , n = 10; moderate COPD (GOLD stage 2), 0.092 ± 0.016 , n = 18, $P < 0.01$ vs. NSC; severe COPD (GOLD stage 3), 0.13 ± 0.024 , n = 6, $P < 0.001$ vs. NSC; very severe COPD (GOLD stage 4), 0.15 ± 0.016 , n = 10, $P < 0.001$ vs. NSC, $P < 0.05$ vs. SC, $P < 0.05$ vs. Stage 1). PI3K-δ (p110δ isoform) mRNA was significantly up-regulated in patients with COPD (S3–4) compared with those of healthy volunteers (PI3K-δ/GNB2L1 ratio, NSC 0.079 ± 0.017 , n = 17; SC, 0.085 ± 0.016 , n = 14; mild COPD (GOLD stage 1), 0.11 ± 0.024 , n = 10; moderate COPD (GOLD stage 2), 0.12 ± 0.016 , n = 18; severe COPD (GOLD stage 3), 0.17 ± 0.062 , n = 6; very severe COPD (GOLD stage 4), 0.19 ± 0.0362 , n = 10, $P < 0.05$ vs. NSC) (Figure 2B), whereas PI3K-α (p110α isoform) (data not shown) and PI3K-γ (p110γ isoform) (Figure 2C) were not increased. Furthermore, PI3K-δ was immunoprecipitated from peripheral lung tissue and its activity was measured by production of PIP₃. PI3K-δ activity was also significantly elevated in severe COPD (NSC: 3.1 ± 0.57 pmol, n = 10; SC: 3.9 ± 0.66 pmol, n = 10; mild COPD (Stage 1): 4.40 ± 1.4 pmol, n = 5; moderate COPD (Stage 2): 6.6 ± 0.70 pmol, n = 10; severe COPD (stage 3): 8.1 ± 1.5 pmol, n = 5; very severe COPD (stage 4): 9.0 ± 0.75 pmol, n = 7; $P < 0.001$ vs. NSC) (Figure 2D). In addition, PI3K-δ (p110δ isoform) mRNA expressions in alveolar macrophages were significantly upregulated in exsmokers with COPD compared with those of exsmokers without COPD or healthy volunteers, but there was no difference of PI3K-δ expression between current smokers and exsmokers (see Figures E2A–E2C).

PI3Kδ Plays an Important Role of Corticosteroid Insensitivity under Oxidative Stress

Fluorescence activated cell sorting analysis showed that the oxidant hydrogen peroxide (H₂O₂) concentration dependently increased pAkt, peaking at 200 μM and also peaking at 7 minutes in U937 cells (see Figures E3A and E3B). This result was confirmed in Western blotting (see Figure E3C).

U937 cells pretreated with H₂O₂ had a higher IC₅₀-Dex than those without H₂O₂ (control 147.1 ± 51.0 nM; H₂O₂ $1,739.0 \pm 744.0$ nM, $P = 0.0023$) (see Figure E3D), suggesting that H₂O₂-

TABLE 1. PHARMACOLOGIC EVALUATION OF DEXAMETHASONE SENSITIVITY IN THE PRESENCE OR ABSENCE OF ADD-ON TREATMENT

Group	n	pAkt/Akt Level	Dexamethasone Response	Nontreatment	LY294002 (10^{-6} M)
High pAkt	7	0.15 ± 0.04	EC ₅₀ (nM)	23 ± 9.6	$4.5 \pm 1.2^*$
			E-max (%)	60 ± 5.9	68 ± 5.3
Low pAkt	4	0.025 ± 0.01	EC ₅₀ (nM)	7.8 ± 2.8	16 ± 4.0
			E-max (%)	62 ± 2.6	58 ± 3.3
Group	n	pAkt Level		Nontreatment	Theophylline (10^{-6} M)
High pAkt	8	0.13 ± 0.03	EC ₅₀ (nM)	21 ± 8.4	$4.3 \pm 1.1^*$
			E-max (%)	60 ± 5.1	61 ± 3.9
Low pAkt	6	0.025 ± 0.01	EC ₅₀ (nM)	7.5 ± 2.4	7.3 ± 1.4
			E-max (%)	62 ± 2.1	60 ± 1.9

* $P < 0.05$ vs. nontreatment.

High pAkt, > 0.05 (pAkt/Atk ratio); low pAkt, < 0.05 (pAkt/Atk ratio).

Figure 2. Phosphoinositide-3-kinase (PI3K) activation in chronic obstructive pulmonary disease (COPD). Whole tissue extracts were prepared from peripheral lung tissue of nonsmoking control subjects (NSCs) ($n = 16$), smoking control subjects (SCs) ($n = 13$), COPD stage 1 (mild; $n = 10$), COPD stage 2 (moderate; $n = 18$), COPD stage 3 (severe; $n = 6$), and COPD stage 4 (very severe; $n = 10$) in lysis buffer, and pAkt, total Akt and α -tubulin were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/Western blotting. (A) The ratio of pAkt and total Akt was calculated by measuring band density. (B) The mRNA of PI3K- δ in peripheral lung tissue was measured by the Taqman reverse transcriptase polymerase chain reaction system and transcripts were normalized to that of GNB2L1; $P < 0.05$ versus nonsmoker. (C) PI3K- δ was also immunoprecipitated from lung tissue of NSCs ($n = 10$), SCs ($n = 10$), COPD stage 1 (mild; $n = 5$), COPD stage 2 (moderate; $n = 8$), COPD stage 3 (severe; $n = 5$), and COPD stage 4 (very severe; $n = 7$) and its activity was measured using a PIP₃ assay mass kit. ** $P < 0.01$ or *** $P < 0.001$ versus NSC, # $P < 0.05$ versus SC, \$ $P < 0.05$ versus S1.

treated cells can mimic the steroid insensitivity of COPD cells. Knockdown of PI3K- δ by RNA interference (87% knockdown in mRNA base) in U937 cells resulted in a failure to develop steroid insensitivity (Dex-IC₅₀ with H₂O₂ 39.2 $\pm$ 21.7 nM, vs. baseline 28.0 $\pm$ 7.7 nM), whereas control cells transfected with scrambled oligonucleotides developed the expected corticosteroid insensitivity with H₂O₂ treatment (Dex-IC₅₀: H₂O₂ 213.0 $\pm$ 155.9 nM vs. baseline 20.7 $\pm$ 6.1 nM, $P = 0.016$) (Figure 3A). By contrast, PI3K- γ knockdown cells developed steroid insensitivity with oxidative stress (Dex-IC₅₀ with H₂O₂ 271.6 $\pm$ 158.5 nM vs. baseline 39.2 $\pm$ 21.7 nM, $P = 0.016$) (Figure 3A). We also found a significant reduction of immunoprecipitated HDAC2 activity after H₂O₂ treatment, and the reduction in HDAC2 activity was prevented in PI3K- δ knockdown cells (PI3K- δ KD: untreated 3.5 $\pm$ 0.29 units, H₂O₂ 4.2 $\pm$ 0.51 units; scrambled oligonucleotide: untreated 4.6 $\pm$ 0.49 units, H₂O₂ 2.1 $\pm$ 0.37 units, $P = 0.029$, $n = 4$) (Figure 3B). PI3K- γ knockdown cells still showed reduced HDAC2 activity with oxidative stress cells (PI3K- γ KD: untreated 5.0 $\pm$ 0.44 units,

H₂O₂ 2.4 $\pm$ 0.40 units, $P = 0.029$). This HDAC2 reduction was also reversed by LY294002 completely (see Figure E3E).

Restoration of Corticosteroid Sensitivity by Low-Dose Theophylline *In Vitro*

Although theophylline (Theo 10⁻⁶M; 501.3 $\pm$ 89.9 pg/ml, $n = 16$) did not inhibit IL-8 production in PBMCs from patients with COPD (499.3 $\pm$ 86.4 pg/ml, $n = 16$) (Figure E1C), Theo significantly restored corticosteroid sensitivity in patients with COPD (IC₅₀-Dex 69.7 $\pm$ 10.6 nM with Theo vs. 156.8 $\pm$ 32.6 nM in controls, $P = 0.0128$, $n = 17$) (Figure 4A) as similar to LY (10⁻⁶ M) (Figure 1C). Enhancement of IC₅₀-Dex by Theo, which was calculated as the ratio of IC₅₀-Dex to IC₅₀-Dex with Theo, significantly correlated with that with percentage enhancement by LY (Spearman $r = 0.64$; $P = 0.024$; $n = 12$) (Figure E1F), suggesting that responders to Theo are nearly identical to the responders to LY. Pharmacologic analysis also showed that theophylline reduced EC₅₀ values in pAkt high group, and did not have any impact on E-max as seen in LY-294002 (Table 1).

To investigate an importance of HDAC2 as the target of theophylline on restoration of corticosteroid sensitivity in primary cells, HDAC2 was knocked down in PBMCs obtained from healthy volunteers, and the sensitivity of dexamethasone was measured as shown previously. IC₅₀ value of dexamethasone was significantly increased in HDAC2 knockdown cells, suggesting corticosteroid insensitivity in HDAC2 knockdown cells. Add-on treatment of theophylline (10⁻⁶M) did not restore corticosteroid sensitivity in HDAC2 knockdown cells, suggesting HDAC2 is a prerequisite molecule in theophylline action (Figure 4B). Theophylline also restored corticosteroid sensitivity in U937 cells treated with H₂O₂ and LY294002 (see Figure E3D).

Low-Dose Theophylline Inhibits PI3K- δ Activity *In Vitro*

H₂O₂-induced pAkt production was abolished by LY (10⁻⁶M) and theophylline (10⁻⁶M) in U937 cells ($n = 4$) (Figure 4C). Akt was also phosphorylated in lung tissue of smoking mice and it was inhibited by theophylline treatment (see Figure E4C).

PI3K- δ was immunoprecipitated in cells with or without H₂O₂ treatment. Inhibition by theophylline of isolated PI3K- δ activity was evaluated by a PIP₃ mass assay kit. As shown in Figure 4D, theophylline concentration dependently inhibited activity of PI3K- δ immunoprecipitated from control cells but more potently inhibited the activity of PI3K- δ precipitated from H₂O₂-treated

Figure 3. Inhibition of PI3K- δ reverses corticosteroid insensitivity under oxidative stress. (A) Either PI3K- δ short interference (si) RNA, PI3K- γ siRNA, or scrambled oligonucleotides was transfected to U937 cells using Nucleofector (Amaxa Biosystems, Cologne, Germany). The cells were incubated for 48 hours and treated with 200 μ M of H₂O₂ for 20 minutes. The cells were then stimulated with TNF- α (10 ng/ml) in the presence of dexamethasone and IC₅₀-Dex was calculated as the index of corticosteroid sensitivity. * $P < 0.05$. (B) HDAC2 was also immunoprecipitated from the PI3K knocked-down (KD) U937 cells after H₂O₂ treatment and the activity was measured. ## $P < 0.01$.

Figure 4. Theophylline is able to reverse corticosteroid insensitivity by PI3K δ inhibition. (A) Peripheral blood mononuclear cells (PBMCs) obtained from 16 patients with chronic obstructive pulmonary disease (COPD) were stimulated with tumor necrosis factor (TNF)- α (1 ng/ml) with or without serial concentrations of dexamethasone (Dex, 10^{-11} – 10^{-6} M) in the presence or absence of theophylline (Theo, 10^{-6} M). IC_{50} -Dex values were calculated individually. (B) HDAC2 siRNA was transfected to PBMCs from healthy volunteers and cells were incubated for 24 hours. IC_{50} -Dex values on TNF- α -induced IL-8 production was calculated. (C) U937 cells were stimulated with H₂O₂ for 7 minutes in the presence or absence of theophylline (Theo, 10^{-6} M) or LY294002 (LY, 10^{-6} M) for 30 minutes before H₂O₂, and pAkt level was determined by Western blotting. (D) PI3K- δ was immunoprecipitated from the cells treated with 200 μ M H₂O₂ for 7 minutes or nontreated cells, and PI3K- δ activity was measured by a PIP3 detection kit in the presence or absence of theophylline (10^{-9} – 10^{-3} M).

cells (IC_{50} value 137.3 ± 8.7 μ M for intact PI3K- δ and 2.1 ± 0.12 μ M for H₂O₂-treated PI3K- δ , $n = 3$) (Figure 4E).

Reversal of Corticosteroid Resistance in Cigarette Smoke-exposed Mice *In Vivo* by IC87114 and Theophylline

A/J mice were exposed to cigarette smoke (4%) for 10 days (30 min/d), and then treated with IC87114, a selective PI3K- δ inhibitor, at 20 mg/kg orally twice daily, or dexamethasone (10 mg/kg orally) for 3 days (see Figure E4A). Cigarette smoke exposure had no effect on body weight (air 23.6 ± 3.9 g vs. smoke 24.2 ± 4.4 g). IC87114 showed additive and synergistic inhibition with dexamethasone on alveolar macrophage (Figure 5A) and neutrophil accumulation (Figure 5B) into BAL in cigarette smoke-exposed mice (neutrophils: smoke + vehicle: $1.83 \pm 0.37 \times 10^4$ cells/ml, smoke + dexamethasone alone: $1.50 \pm 0.19 \times 10^4$, smoke + IC87114 alone: $1.16 \pm 0.20 \times 10^4$, smoke + IC87114/dexamethasone: $0.64 \pm 0.06 \times 10^4$, air control: $0.094 \pm 0.013 \times 10^4$ cells/ml) (alveolar macrophage: smoke + vehicle: $25.9 \pm 4.4 \times 10^4$ cells/ml, smoke + dexamethasone alone: $19.2 \pm 1.7 \times 10^4$, smoke + IC87114 alone: $19.8 \pm 2.6 \times 10^4$, smoke + IC87114/dexamethasone: $10.2 \pm 0.53 \times 10^4$, air control: $1.5 \pm 0.2 \times 10^4$ cells/ml). Furthermore, the combination of dexamethasone and LY also significantly inhibited neutrophil and alveolar macrophage accumulation into BAL fluid (Figures 5A and 5B).

Add-on treatment of theophylline on dexamethasone was evaluated in smoking-exposed mice. The number of neutrophils

in BAL fluid was increased by cigarette smoke exposure (air $0.02 \pm 0.02 \times 10^4$ cells/ml vs. smoke $0.34 \pm 0.13 \times 10^4$ cells/ml) (Figure 5C) and alveolar macrophages (air $6.9 \pm 2.3 \times 10^4$ cells/ml vs. smoke $24.2 \pm 4.4 \times 10^4$ cells/ml (see Figure E3B)). The neutrophil accumulation was not inhibited by either Dex or Theo given alone, but combination treatment with both drugs significantly inhibited the neutrophil accumulation by 86.8% (Figure 5C) and the number of alveolar macrophages was also inhibited 90.2% only by combinations (smoke with both drugs $11.1 \pm 2.1 \times 10^4$ cells/ml vs. smoke with no treatment $24.2 \pm 4.4 \times 10^4$ cells/ml, $P < 0.05$) (see Figure E4B).

Immunoprecipitated HDAC2 activity was also significantly reduced by cigarette smoke and restored by theophylline (air 4.6 ± 0.43 units, smoke 3.2 ± 0.35 units, smoke + dexamethasone 3.5 ± 0.20 units, smoke + theophylline 4.7 ± 0.47 units, smoke + theophylline dexamethasone combination 5.7 ± 0.84 units) (Figure 5D).

DISCUSSION

Several studies have shown that the inflammation in COPD lungs is corticosteroid-insensitive clinically (3) and pulmonary cells, such as macrophages, are also reported to be corticosteroid-insensitive *in vitro* (16). The present study demonstrated that PBMCs obtained from patients with COPD were also less corticosteroid-sensitive than cells from age-matched healthy volunteers and healthy smokers (Figure 1A). Patients with more severe disease who had worse lung function had a greater degree of steroid insensitivity (Figure 1C). Thus, not only resident cells in lung, but also circulating cells are corticosteroid insensitive in COPD. The molecular mechanisms of corticosteroid insensitivity in COPD are complex, but we have previously reported that a dominant molecular mechanism is a reduction of HDAC2, which causes glucocorticoid receptor hyperacetylation and failure to switch off activated inflammatory genes, such as IL-8 (3, 17).

In this study HDAC2 activity was reduced by treatment of H₂O₂ as a source of oxidative stress, and this reduction was reversed by LY-294002, a nonselective PI3K inhibitor. LY-294002 also reversed corticosteroid insensitivity under conditions of oxidative stress, suggesting that PI3K activation may reduce HDAC2 activity, thereby resulting in corticosteroid insensitivity. Because HDAC is a phosphoprotein and is reported to be phosphorylated by casein kinase-2, resulting in enhanced HDAC activity (18), PI3K may target different sites on HDAC2.

There is increasing evidence that oxidative stress plays a major role in amplifying inflammation in COPD lungs (19). Our results demonstrate that oxidative stress increased the phosphorylation (Ser⁴⁷³) of Akt/PKB, which is a direct target of PI3K and therefore functions as a surrogate marker for PI3K activation (see Figures E3A–E3C) as reported previously in different cell systems (20, 21). Phosphorylated Akt levels tended to be increased in PBMCs of patients with COPD (see Figure E1D) and were significantly increased in peripheral lung tissue obtained from patients with COPD compared with normal subjects (Figure 2A). Similarly, cigarette smoke-exposed mice also showed an increased level of pAkt in peripheral lung tissue (see Figure E4D). Thus, PI3K is clearly activated in patients with COPD or under conditions of oxidative stress and cigarette exposure.

PI3Ks are subdivided into four classes (IA, IB, II, and III), which possess lipid kinase activity, and the kinases largely responsible for PIP₃ are class I-PI3Ks (9). Class I-PI3K contains four isoforms of the catalytic subunit (p110 α , p110 β , p110 γ , and p110 δ). We used IC87114, a selective PI3K- δ inhibitor, and found this compound also inhibited neutrophil accumulation with dexamethasone, although neither dexamethasone nor IC87114 alone were effective (Figures 5A and 5B). Further-

Figure 5. Reversal of corticosteroid insensitivity by theophylline and PI3K- δ inhibition in smoking mice. A/J mice were exposed to cigarette smoke (4%) for 30 minutes per day for 10 days. IC87114 (20 mg/kg orally: IC), dexamethasone (5 mg/kg orally: Dex), LY294002 (20 mg/kg orally: LY), a combination of these treatments, or PEG400 as control were administered twice daily for 3 days for five animals each following the last cigarette exposure. The number of alveolar macrophages (A) and neutrophils (B) in bronchoalveolar lavage fluid was calculated; * $P < 0.05$, ** $P < 0.01$ versus smoke control, ## $P < 0.01$ between air and smoke control. (C) Theophylline (10 mg/kg orally: Theo), dexamethasone (10 mg/kg orally: Dex), a combination of these treatments, or 0.5% carboxymethylcellulose as control were therapeutically administered once daily for 3 days following the last cigarette exposure. The number of neutrophils in bronchoalveolar lavage was shown. (D) HDAC2 was immunoprecipitated in nuclear-rich extract from lung tissue of the mice treated with indicated compounds, and its activity was measured by fluorescent-based HDAC activity assay kit. * $P < 0.05$, ** $P < 0.01$ versus smoke control.

more, the inhibitory action of the IC87114-dexamethasone combination was similar to that of combination of a nonselective inhibitor, LY294002 and dexamethasone. These strongly suggest that the PI3K- δ isoform is involved in corticosteroid insensitivity in the smoking murine model. We also showed here that PI3K- δ mRNA activity was significantly increased in peripheral lung tissue from patients with COPD and alveolar macrophages (Figures 2B and 2D, and Figure E2). Furthermore, PI3K- δ knockdown by RNA interference resulted in protection against corticosteroid insensitivity and reduced HDAC2 activity after exposure to oxidative stress, whereas PI3K- γ knockdown had no such effect (Figures 3A and 3B). We also recently reported that PI3K δ knockout mice did not develop corticosteroid sensitivity by smoke exposure (22), and this agrees with the results shown here.

PI3K- δ is reported to regulate neutrophil activation (23), trafficking (24), and directional movement (25). Mice deficient in both PI3K- γ and PI3K- δ have a block in T-lymphocyte development and a survival defect (26). A PI3K- δ selective inhibitor has also been reported to reduce allergic airway inflammation (27, 28), indicating that it may play a role in lung inflammation. However, LY-294002 did not inhibit TNF- α -induced IL-8 production in PBMCs from patients with COPD or oxidative stress-treated U937 cells. PI3K- δ knockdown cells showed only 20–30% inhibition of TNF- α -induced IL-8 production, even though it completely prevented the development of steroid insensitivity. IC87114 alone was also ineffective on IL-8 production in BAL of smoking mice (data not shown). This suggests that the antiinflammatory effect of PI3K- δ inhibition is limited, whereas it plays a critical role in restoration of corticosteroid sensitivity. By contrast, PI3K- γ knockdown has a greater antiinflammatory effect but no effect in restoring corticosteroid sensitivity. This suggests that PI3K- δ inhibition has the potential to enhance the antiinflammatory efficacy of corticosteroids and to reverse corticosteroid insensitivity in such diseases as COPD.

Theophylline is a drug that has long been used for the treatment for asthma and COPD as a bronchodilator. The clinical use of theophylline has now declined because of the high frequency of side effects, such as headache and nausea, which are

mediated by PDE inhibition, and cardiac arrhythmias and seizures as a result of adenosine receptor antagonism at the concentrations of drug needed for bronchodilation (9). However, theophylline shows antiinflammatory effects at lower plasma concentrations (29), and an add-on treatment of low-dose theophylline enhances the effects of corticosteroids in patients with asthma (30). As shown in Figures 4A and E3D, a low concentration of theophylline (10^{-6} M, which corresponds to a plasma concentration of 0.18 mg/L), which does not produce either PDE inhibition or adenosine antagonism, was able to restore corticosteroid sensitivity in PBMCs from patients with COPD and in H_2O_2 -treated cells. We have previously reported that theophylline enhances HDAC2 activity as a molecular basis for reversing corticosteroid insensitivity in COPD cells. We have now confirmed this in HDAC2 knockdown PBMCs from healthy volunteers (Figure 4A). However, the molecular mechanism for the enhancement of HDAC2 activity by low concentrations of theophylline was previously unknown.

Theophylline has previously been reported to inhibit the δ -subunit of PI3K at lower concentrations than the other isoenzymes (8). Our data confirm that theophylline inhibits isolated intact PI3K- δ with a similar potency (Figure 4E). In addition, theophylline potently inhibited oxidative stress-induced pAkt expression and the conversion of PIP_2 to PIP_3 under conditions of oxidative stress (Figures 4C and 4D).

However, there is a discrepancy between the reported concentration of theophylline for PI3K- δ inhibition (IC_{50} 75 μ M) (8) and the concentration of theophylline to restore corticosteroid sensitivity in PBMCs from COPD-U937 cells under oxidative stress (10^{-6} M; 1 μ M). As shown in Figure 5C, theophylline was 65 times more sensitive in PI3K- δ immunoprecipitated from H_2O_2 -treated cells (2.1 μ M) than PI3K- δ from intact cells (137 μ M), indicating that oxidative stress markedly enhances the potency of theophylline as an inhibitor of PI3K- δ . PI3K is reported to be an oxidized protein, but this modification seems to downregulate its enzyme activity, rather than increasing it. H_2O_2 stimulation is also reported to activate PI3K through tyrosine phosphorylation of the p110 but not the p85 subunit of PI3K in DT40 cells (a chicken B-cell line) (31).

Although the molecular mechanism is still unclear, post-translational modification of PI3K- δ may be involved in the marked increase in sensitivity to theophylline. This would have the effect of making theophylline more effective under conditions of oxidative stress, as in COPD.

In cigarette smoke-exposed mice, which are now commonly used as an experimental model of COPD, low-dose theophylline (plasma concentration 4.0 ± 0.90 mg/L 1 h after treatment of 10 mg/kg orally administered theophylline) clearly enhanced the antiinflammatory effect of dexamethasone, with concomitant restoration of HDAC2 activity (Figures 5C and 5D). The enhancement of dexamethasone efficacy on inflammatory cell accumulation was also seen in mice treated with IC87114, a selective PI3K- δ inhibitor (Figures 5A and 5B). We used a therapeutic dosing strategy with administration of theophylline or IC87114 following repeated cigarette exposure that reflects the expected clinical use of the drug once inflammation is established.

Our results strongly suggest that inhibition of oxidative stress-activated PI3K- δ would be an effective therapeutic strategy in patients with COPD with corticosteroid insensitivity. Because the side effects of theophylline are mediated by different molecular mechanisms, this suggests that it may be possible to find alternative theophylline-like drugs that avoid the side effects of theophylline or to develop selective PI3K- δ inhibitors, such as IC87114, for reversing corticosteroid insensitivity in COPD. Other corticosteroid-insensitive diseases, such as severe asthma, inflammatory bowel disease, and rheumatoid arthritis, may have similar mechanisms of corticosteroid insensitivity, because oxidative stress is increased in these diseases and this suggests that theophylline and selective PI3K- δ inhibitors may also be effective.

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References

1. Lopez AD, Murray CC. The global burden of disease, 1990–2020. *Nat Med* 1998;4:1241–1243.
2. Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J* 2003;22:672–688.

3. Barnes PJ, Ito K, Adcock IM. Corticosteroid resistance in chronic obstructive pulmonary disease: inactivation of histone deacetylase. *Lancet* 2004;363:731–733.
4. Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, Yates JC, Vestbo J. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med* 2007;356:775–789.
5. Vestbo J, Sorensen T, Lange P, Brix A, Torre P, Viskum K. Long-term effect of inhaled budesonide in mild and moderate chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* 1999;353:1819–1823.
6. Cosio BG, Tsaprouni L, Ito K, Jazrawi E, Adcock IM, Barnes PJ. Theophylline restores histone deacetylase activity and steroid responses in COPD macrophages. *J Exp Med* 2004;200:689–695.
7. Ito K, Lim S, Caramori G, Cosio B, Chung KF, Adcock IM, Barnes PJ. A molecular mechanism of action of theophylline: induction of histone deacetylase activity to decrease inflammatory gene expression. *Proc Natl Acad Sci U S A* 2002;99:8921–8926.
8. Ito K, Ito M, Elliott WM, Cosio B, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, et al. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med* 2005;352:1967–1976.
9. Barnes PJ. Theophylline in chronic obstructive pulmonary disease: new horizons. *Proc Am Thorac Soc* 2005;2:334–339.
10. Foukas LC, Daniele N, Ktori C, Anderson KE, Jensen J, Shepherd PR. Direct effects of caffeine and theophylline on p110 delta and other phosphoinositide 3-kinases: differential effects on lipid kinase and protein kinase activities. *J Biol Chem* 2002;277:37124–37130.
11. Ito K, Caramori G, Adcock IM. Therapeutic potential of phosphatidylinositol 3-kinase inhibitors in inflammatory respiratory disease. *J Pharmacol Exp Ther* 2007;321:1–8.
12. To Y, Failla M, Ito M, Adcock IM, Barnes PJ, Ito K. Theophylline restores corticosteroid sensitivity in COPD by inhibition of PI3-kinase-delta [abstract]. *Proc Am Thorac Soc* 2007;2:A45.
13. The Global Initiative for Chronic Obstructive Lung Disease (GOLD). 2008 (accessed December 5, 2009). Available from: <http://www.goldcopd.com>.
14. Cosio BG, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF, Adcock IM. Histone acetylase and deacetylase activity in alveolar macrophages and blood monocytes in asthma. *Am J Respir Crit Care Med* 2004;170:141–147.
15. Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, Kodama Y, Mori T, Goto S, Takahashi K, et al. Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. *Am J Respir Crit Care Med* 2006;174:530–537.
16. Culpitt SV, Rogers DF, Shah P, De MC, Russell RE, Donnelly LE, Barnes PJ. Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;167:24–31.
17. Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, Adcock IM. Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J Exp Med* 2006;203:7–13.
18. Tsai SC, Seto E. Regulation of histone deacetylase 2 by protein kinase CK2. *J Biol Chem* 2002;277:31826–31833.
19. Bowler RP, Barnes PJ, Crapo JD. The role of oxidative stress in chronic obstructive pulmonary disease. *COPD* 2004;1:255–277.
20. Baregamian N, Song J, Jeschke MG, Evers BM, Chung DH. IGF-1 protects intestinal epithelial cells from oxidative stress-induced apoptosis. *J Surg Res* 2006;136:31–37.
21. Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Kuwabara M. Redox regulation of PI3K/Akt and p53 in bovine aortic endothelial cells exposed to hydrogen peroxide. *Antioxid Redox Signal* 2003;5:713–722.
22. Marwick JA, Caramori G, Stevenson CS, Casolari P, Jazrawi E, Barnes PJ, Ito K, Adcock IM, Kirkham PA, Papi A. Inhibition of PI3Kdelta restores glucocorticoid function in smoking-induced airway inflammation in mice. *Am J Respir Crit Care Med* 2009;179:542–548.
23. Sadhu C, Dick K, Tino WT, Staunton DE. Selective role of PI3K delta in neutrophil inflammatory responses. *Biochem Biophys Res Commun* 2003;308:764–769.
24. Puri KD, Doggett TA, Douangpanya J, Hou Y, Tino WT, Wilson T, Graf T, Clayton E, Turner M, Hayflick JS, et al. Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue. *Blood* 2004;103:3448–3456.

25. Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J Immunol* 2003;170:2647–2654.
26. Webb LM, Vigorito E, Wymann MP, Hirsch E, Turner M. Cutting edge: T cell development requires the combined activities of the p110gamma and p110delta catalytic isoforms of phosphatidylinositol 3-kinase. *J Immunol* 2005;175:2783–2787.
27. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J* 2006;20:455–465.
28. Lee KS, Park SJ, Kim SR, Min KH, Jin SM, Puri KD, Lee YC. Phosphoinositide 3-kinase-delta inhibitor reduces vascular permeability in a murine model of asthma. *J Allergy Clin Immunol* 2006;118:403–409.
29. Culpitt SV, De MC, Russell RE, Donnelly LE, Rogers DF, Barnes PJ. Effect of theophylline on induced sputum inflammatory indices and neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;165:1371–1376.
30. Lim S, Jatakanon A, Gordon D, Macdonald C, Chung KF, Barnes PJ. Comparison of high dose inhaled steroids, low dose inhaled steroids plus low dose theophylline, and low dose inhaled steroids alone in chronic asthma in general practice. *Thorax* 2000;55:837–841.
31. Qin S, Chock PB. Implication of phosphatidylinositol 3-kinase membrane recruitment in hydrogen peroxide-induced activation of PI3K and Akt. *Biochemistry* 2003;42:2995–3003.