Peroxisome proliferator-activated receptor-γ inhibits cigarette smoke solution-induced mucin production in human airway epithelial (NCI-H292) cells

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THE CLINICAL HALLMARKS of chronic bronchitis are cough and excessive mucus production (40, 46). The main etiologic factor for chronic bronchitis is cigarette smoke. Exposure to cigarette smoke is reported to induce goblet cell hyperplasia and mucus production. Mucin synthesis in airways has been reported to be regulated by the EGFR system. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the ligand-activated nuclear receptor superfamily. PPAR-γ is implicated in anti-inflammatory responses, but mechanisms underlying these varied roles remain ill-defined (23). Recently, reports have shown that upregulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) might be one of the mechanisms through which PPAR-γ agonists exert their anti-inflammatory actions. However, no data are available on the role of PPAR-γ in smoke-induced mucin production. In this study, we investigated the effect of PPAR-γ agonist (rosiglitazone) on smoke-induced mucin production. In this study, we investigated the effect of PPAR-γ agonist (rosiglitazone) on smoke-induced mucin production.

MATERIALS AND METHODS

Preparation of cigarette smoke solution. Standard research cigarettes (code 2R1, produced by the University of Kentucky Tobacco and Health Research Foundation) were used in this study. Cigarette smoke solution was prepared as described previously (46). In brief, cigarette smoke was withdrawn into a polypropylene syringe (50 ml) at a rate of one puff/min and bubbled slowly into 50 ml of RPMI 1640 medium containing 50 mM HEPES buffer. The smoke solution was then titrated to pH 7.4 and used immediately after preparation.

Cell culture. NCI-H292 cells, a human pulmonary mucoid carcinoma cell line, were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM) at 37°C in a humidified 5% CO2 water-jacketed incubator. Six-well culture plates were used to culture the cells. When confluent, cells were incubated for 1 h with cigarette smoke solution. The cells were then washed and incubated with fresh medium alone. Experiments were terminated at preselected times (for protein, 24 h). For the controls, cells were incubated with medium alone for the same time periods. In inhibition studies, NCI-H292 cells were pretreated with wortmannin (20 nM; Sigma Aldrich, St. Louis, MO), LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, 30 μM; Sigma Aldrich], and tyrphostin AG-1478 (10 μM, Calbiochem) 30 min before the cigarette smoke solution was delivered. To evaluate the effect of PPAR-γ agonist (rosiglitazone) 30 min before the cigarette smoke solution was delivered.

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Measurement of TNF-α. NCI-H292 cells were incubated with cigarette smoke solution or were treated in culture medium with various concentrations of rosiglitazone (0.1, 1 μM) 30 min before they were incubated in cigarette smoke solution. The TNF-α level was determined by ELISA (Sigma) from supernatants harvested at 24 h.

Immunofluorescent staining of MUC5AC protein. NCI-H292 cells were incubated with cigarette smoke solution or were treated in culture medium with wortmannin, LY-294002, AG-1478, or TNF-α with cigarette smoke solution or were treated in culture medium with washed three times with PBS, and 100 μl of each sample were then incubated with bicarbonate-carbonate buffer (50 μl) at 40°C in a 96-well plate (Maxisorp Nunc; Fisher Scientific, Santa Clara, CA) until dry. The plates were then rewarmed three times with PBS and blocked with 2% bovine serum albumin, fraction V (Sigma Aldrich) with PBS at multiple dilutions, and 50 μl of each sample were then incubated with monoclonal antibody (infiximab). Infiximab was purchased from a pharmaceutical supplier (Schering Korea, Seoul, Korea). MUC5AC protein was measured. In brief, cell culture supernatants were prepared with PBS at multiple dilutions, and 50 μl of each sample were then incubated with bicarbonate-carbonate buffer (50 μl) at 4°C in a 96-well plate (Maxisorp Nunc; Fisher Scientific, Santa Clara, CA) until dry. The plates were then rewarmed three times with PBS and blocked with 2% bovine serum albumin, fraction V (Sigma Aldrich) diluted with PBS containing 0.05% Tween 20. After 1 h, the wells were washed three times with PBS, and 100 μl of horseradish peroxidase-anti-mouse IgG conjugate (1:10,000) were dispensed into each well. After a further 1 h, the plates were washed three times with PBS. Color reaction was developed using 3,3',5,5'-tetramethylbenzidine peroxidase solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and stopped with 2 N H2SO4. Absorbance was read at 450 nm. Each experiment was repeated at least five times. Mucin of the submandibular gland was used as a positive ELISA control and naïve medium (RPMI+10% FBS, never exposed to cells) was used as a negative control.

Western blotting. NCI-H292 cells were harvested and washed twice with PBS. The cell pellet was lysed with mammalian protein extraction reagent lysis buffer (Pierce, Rockford, IL) and 1:100 dilution of protease inhibitors (Pierce). To remove insoluble materials, cell lysates were centrifuged at 14,000 rpm for 5 min, and 1 vol of Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 4 mg/ml bromphenol blue, and 125 mM Tris-HCl, pH 6.8; Bio-Rad Laboratories) was added to the supernatant. After incubation at 95°C for 5 min, total proteins were separated by SDS-PAGE in 10% acrylamide gels. The running gel was electrotransferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories), which was blocked with 5% fat-free milk in Tris-buffered saline/0.05% Tween 20 for 1 h at room temperature. Membranes were incubated with anti-EGFR (Ab-2) monoclonal antibody (EGFR, 1:1,000, 100 μg/ml; Oncogene), anti-phospho-specific EGFR monoclonal antibodies (EGFR-p, 1:1,000, 1 μg/ml; Calbiochem), anti-PTEN (1:800, Santa Cruz Biotechnology), and anti-pAkt (1:1,000, Calbiochem). The membranes were stripped and rebotted with monoclonal anti-β-actin antibody (Sigma Aldrich) to verify equal loading of protein in each lane. The membranes were incubated with horseradish peroxidase-linked secondary antibody (Vector, Burlingame, CA) for 1 h at room temperature. Proteins were detected using the Supersignal West picochemiluminescent substrate kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

Statistics. All data are expressed as means ± SE. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. Scheffé’s F-test was used to correct for multiple comparisons when statistical significances were identified in the ANOVA. P < 0.05 for the null hypothesis was accepted as indicating a statistically significant difference.

RESULTS

Cigarette smoke induces EGFR tyrosine phosphorylation and MUC5AC protein synthesis. Because the activation of EGFR leads to MUC5AC synthesis, we examined the effect of cigarette smoke solution on activation of the EGFR tyrosine kinase, and AG-1478, a potent and selective chemical inhibitor of EGFR tyrosine kinase activity, was used to block EGFR signal transduction. As expected, pretreatment (30 min) with cigarette smoke solution induced EGFR-specific tyrosine phosphorylation, and AG-1478 inhibited the cigarette smoke solution-induced increase of p-EGFR levels (Fig. 1). The concentrations were determined from previous studies (47). NCI-H292 cells were treated with various doses of cigarette smoke. Cigarette smoke solution caused increase in MUC5AC protein synthesis dose dependently (Fig. 2).

Rosiglitazone inhibits cigarette smoke-induced TNF-α production from NCI-H292 cells. TNF-α can induce EGFR expression, resulting in MUC5AC production as noted in previous studies (45). For the role of TNF-α in cigarette smoke-induced mucin production, we used a TNF-α monoclonal antibody (infiximab) that selectively blocks the effects of TNF-α and measured MUC5AC production. Cigarette smoke-induced MUC5AC protein synthesis was inhibited by infiximab dose dependently (Fig. 3). Therefore, we measured TNF-α levels in cigarette smoke-stimulated NCI-H292 cells. NCI-H292 cells were treated with cigarette smoke solution and rosiglitazone. TNF-α levels were measured in the culture supernatants of the cells. Cigarette smoke solution increased TNF-α production in NCI-H292 cells, whereas rosiglitazone had an inhibitory effect on TNF-α production in cigarette smoke-stimulated NCI-H292 cells in a dose-dependent fashion (Fig. 4).

Effect of rosiglitazone, AG-1478, and P13K inhibitors on MUC5AC production in NCI-H292 cells. To test whether the cigarette smoke-induced MUC5AC production is related to the EGFR and Akt/P13K pathway, cells were incubated with EGFR tyrosine kinase inhibitor, selective P13K inhibitors (wortmannin, LY-294002), and rosiglitazone. Cigarette smoke-induced MUC5AC synthesis was inhibited by pretreatment with EGFR tyrosine kinase inhibitor and selective P13K inhibitors. Furthermore, pretreatment of the cells with PPARY agonist (rosiglitazone) prevented MUC5AC protein synthesis, in a dose-dependent fashion (Fig. 5). These results indicate that smoke-induced MUC5AC mucin production is related, at least in part, to the Akt/P13K pathway.

![Fig. 1. Tyrosine phosphorylation of epidermal growth factor receptor (EGFR) induced by cigarette smoke was inhibited by EGFR tyrosine kinase inhibitor (AG-1478). The cells were pretreated with or without AG-1478 (10 μM) for 30 min and then treated with vehicle or cigarette smoke solution for 1 h. After lysis, EGFR was immunoprecipitated with antiphosphorylated EGFR antibody (top) and with anti-EGFR antibody (bottom). EGFR expression was not significantly different for the different lyses, thus indicating equal protein loading. p, Phosphorylated.](http://ajplung.physiology.org/)

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Rosiglitazone increases PTEN expression with concomitant downregulation of pAkt expression. Because rosiglitazone inhibited the MUC5AC mucin production, we examined whether inhibited mucin production is related to PTEN expression. PTEN, which antagonizes the action of PI3K by dephosphorylation of phosphatidylinositol 3,4,5-triphosphate (PIP3) has been suggested as a possible mechanism of anti-inflammatory action of PPAR-\(\gamma\) agonist (37). Pretreatment of NCI-H292 cells with rosiglitazone increased PTEN expression, which was reduced by cigarette smoke solution. We examined activated Akt (p-Akt), which cannot activate the subsignal pathway while under the PTEN activation state. Cigarette smoke-induced TNF-\(\alpha\) secretion was inhibited by rosiglitazone dose dependently in NCI-H292 cells. The cells were pretreated with or without rosiglitazone (0.1 \(\mu\)M/1 \(\mu\)M) for 30 min and then stimulated with vehicle or cigarette smoke solution for 1 h. We used ELISA to generate data, which are expressed as mean values ± SE (n = 5, *P < 0.01 compared with control, **P < 0.05, †P < 0.01 compared with 9 puffs of cigarette smoke solution alone).

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duced activated Akt expression was inhibited by pretreatment with rosiglitazone (Fig. 6).

**DISCUSSION**

Our study examined the effect of PPAR-γ agonist (rosiglitazone) on mucin production in airway epithelium by using NCI-H292 cells. Delivery of the rosiglitazone to cigarette smoke-induced NCI-H292 cells decreased MUC5AC protein expression with increased PTEN expression. As a result of such increases in the expression of PTEN, the PI3K/Akt pathway was inhibited, and inhibition of Akt pathway abrogated MUC5AC mucin production. To our knowledge, this report is the first to describe this effect of rosiglitazone on cigarette smoke-induced mucin production.

Chronic bronchitis remains one of the most common chronic airway inflammatory diseases and is associated with epithelial metaplasia and mucus production. Various mediators, such as transforming growth factor (TGF)-β, granulocyte-macrophage colony-stimulating factor, EGF, and TNF-α have been reported to regulate airway inflammation. Exposure to cigarette smoke activates an inflammatory cascade in the airway epithelium, resulting in the production of a number of potent cytokines and chemokines, with accompanying damage to the lung epithelium, increased permeability, and recruitment of macrophages and neutrophils to the airway (1). Upregulation of cytokine gene expression in epithelial cells has been linked to the activation of specific signaling pathways. EGFR activation also plays a major role in modulating mucus production in airway epithelium. Takeyama et al. (46) reported that exposure to cigarette smoke upregulated EGFR mRNA expression and induced EGFR-specific tyrosine phosphorylation, resulting in upregulation of MUC5AC mucin mRNA and protein production. EGFR activation may involve two pathways, namely, ligand-dependent and ligand-independent EGFR tyrosine phosphorylation (6). In ligand-dependent EGFR tyrosine phosphorylation, EGFR ligands (EGF or TGF-α) bind to EGFRs in the extracellular domain and activate them, whereas, in ligand-independent EGFR tyrosine phosphorylation, EGFR tyrosine phosphorylation occurs in the absence of exogenous EGFR ligands. Ligand-independent EGFR phosphorylation is reported in response to oxidative stress that can be produced by cigarette smoke and by activated neutrophils (20, 44, 46). Upon activation, these receptors undergo dimerization and tyrosine autophosphorylation, followed by recruitment and activation of signaling molecules that contain MAPK and Akt, resulting in airway epithelial proliferation and mucus hypersecretion (3, 6, 48). Previous studies reported that the MAPK pathway is mainly involved in mucin production induced by a variety of stimuli (15). However, in recent years, a second pathway, downstream of PI3K and Akt, has become a focus of interest (3, 48). The activated PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-biphosphate to phosphatidylinositol-3,4,5-triphosphate (9). This phosphorylation then stimulates the catalytic activity of Akt. Active Akt phosphorylates IκB kinase-α (IKKα), which promotes activation of its heterodimeric partner, IKKB, in the IKKα/IKKβ complex (25, 36). The IKK complex phosphorylates IκB, thereby promoting its dissociation from NF-κB (5). This process allows NF-κB to enter the nucleus, where it activates cytokines, adhesion molecules and other target genes (10). NF-κB has been shown to play an important role in mucus production (11, 31). Thus PI3K/Akt signaling may render cells produce mucin. In the present study, our results show that exposure of airway epithelial cells to cigarette smoke activates EGFR tyrosine phosphorylation, causing MUC5AC mucin synthesis in NCI-H292 cells (Figs. 1 and 2). The Akt phosphorylation is increased, while the PTEN protein expression is reduced, and Akt pathway inhibitor LY-294002 suppressed MUC5AC production in cigarette smoke solution-treated NCI-H292 cells.

PPARs have been reported to regulate inflammatory responses (13). Three subtypes of PPARs (α, β, and γ) have been identified (16). One of the natural ligands for PPAR-γ is 15-deoxy-Δ-prostaglandin J2, the major metabolite of prostaglandin D2 (17). Ligand-induced activation of PPAR results in heterodimerization of the receptor with the retinoid X receptor (RXR) and subsequent binding to specific peroxisome proliferator-responsive elements located within the promoter region of target genes (27). PPAR-γ has been shown to play a major role in regulating adipocyte differentiation and glucose homeostasis. One group of synthetic PPAR-γ-agonist ligands is rosiglitazone, a group of drugs used in the treatment of diabetic patients (49). In addition, there is a growing body of evidence that suggests that PPAR-γ agonists may exert anti-inflammatory action in vivo (2, 30, 34), and some reports have demonstrated that ligand-activated PPAR-γ can downregulate NF-κB transcription (12, 38). Therefore, inhibition of NF-κB activation by PPAR-γ agonist may well reduced expression of proinflammatory mediators (TNF-α, ICAM-1, etc.). A possible clue to the mechanism of PPAR-γ anti-inflammatory action has been provided by the observation that expression of PTEN, which antagonizes PI3K-mediated signaling, is directly upregulated by PPAR-γ (37). PPAR-γ is able to bind two PPAR response elements in genomic sequence upstream of PTEN, and antisense-mediated disruption of PPAR-γ expression prevented the upregulation of PTEN. The PTEN gene constitutes the NH₂-terminal catalytic domain and a dual-specificity phosphatase (32). It is known that PTEN plays critical roles not only in suppressing cancer but also in embryonic development, cell
migration, cell signaling, and apoptosis (4, 14, 21, 43). PTEN appears to serve as a hub or switch point that links complex signaling pathways. Expression of PTEN has opposite effects, such as blocking downstream signaling of EGFR including the Ras/MEK/MAPK cascade and PI3K/Akt signaling (21, 22, 28, 51). The activation of PTEN function results in a decrease of PIP3 concentration, which in turn leads to Akt inactivation. This would appear to suggest that the anti-inflammatory function of PTEN is exerted through the negative regulation of the PI3K/Akt cell signaling pathway (43). Our results show that administration of rosiglitazone results in increase of PTEN expression. The Akt phosphorylation that is increased by cigarette smoking solution is significantly reduced by the administration of rosiglitazone. Together, these findings suggest that PPAR-γ agonist may reduce smoking solution-induced MUC5AC synthesis by increasing PTEN expression.

In conclusion, in our study of cigarette smoke-induced airway epithelial cells exposed to PPAR-γ agonist, decreased inflammatory cytokine (TNF-α) and MUC5AC mucin synthesis was observed, which was shown to be associated with the inhibition of PI3K/Akt pathway (Fig. 7). These findings thus represent the first evidence for a PPAR-γ-dependent and therapeutically significant inhibition of cigarette smoke-induced mucin production. Therefore, activation of PTEN and inhibition of PI3K/Akt by PPAR-γ agonist are proposed as a potential therapy for cigarette smoke-induced mucin secretory diseases. Most patients with chronic obstructive pulmonary disease (COPD) have mucus hypersecretion. Overproduced mucus plugs the airways and contributes to exacerbations of COPD. Presently, there is no effective therapy to relieve the symptoms and to halt the progression of this disease. Our results provide a strategy for therapy by recovery of PTEN expression.

GRANTS
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REFERENCES


