Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke

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Takeyama, Kiyoshi, Birgit Jung, Jae Jeong Shim, Pierre-Regis Burgel, Trang Dao-Pick, Iris F. Ueki, Ursula Protin, Peer Kroschel, and Jay A. Nadel. Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. Am J Physiol Lung Cell Mol Physiol 280: L165–L172, 2001.—Mucus hypersecretion from hyperplastic airway goblet cells is a hallmark of chronic obstructive pulmonary disease (COPD). Although cigarette smoking is thought to be involved in mucus hypersecretion in COPD, the mechanism by which cigarette smoke induces mucus overproduction is unknown. Here we show that activation of epidermal growth factor receptors (EGFR) is responsible for mucin production after inhalation of cigarette smoke in airways in vitro and in vivo. In the airway epithelial cell line NCI-H292, exposure to cigarette smoke upregulated the EGFR mRNA expression and induced activation of EGFR-specific tyrosine phosphorylation, resulting in upregulation of MUC5AC mRNA and protein production, effects that were inhibited completely by selective EGFR tyrosine kinase inhibitors (BIBX1522, AG-1478) and that were decreased by antioxidants. In vivo, cigarette smoke inhalation increased MUC5AC mRNA and goblet cell production in rat airways, effects that were prevented by pretreatment with BIBX1522. These effects may explain the goblet cell hyperplasia that occurs in COPD and may provide a novel strategy for therapy in airway hypersecretory diseases.

synthesis of mucin MUC5AC at both mRNA and protein levels in airway epithelial cells in vitro and causes mucin MUC5AC production and goblet cell metaplasia in rats in vivo (28).

Oxidative stress also has been shown to cause EGFR activation, which results in stimulation of mucin synthesis in airway epithelial cells (29). From these findings, we hypothesized that cigarette smoke, a major exogenous source of oxidative stress, induces mucin synthesis and subsequent goblet cell production via an EGFR signaling pathway. To examine this hypothesis, we tested whether cigarette smoke causes mucin MUC5AC synthesis, and, if so, whether EGFR activation is involved. We performed in vitro studies in bronchial epithelial (NCI-H292) cells, which are known to produce MUC5AC mucins, and in vivo studies in specific pathogen-free rats (a species that normally has few goblet cells constitutively). Here we show that cigarette smoke upregulates EGFR expression and induces mucin synthesis via EGFR activation in the airway epithelium in vitro and in vivo. Inhibition of the EGFR signaling pathway may be useful therapeutically in preventing the hypersecretion that occurs in chronic obstructive pulmonary disease (COPD).

MATERIAL AND METHODS

In Vitro Studies

Preparation of cigarette smoke solution. Standard research cigarettes (code 2R1, produced for the University of Kentucky Tobacco and Health Research Foundation) were used in the study. Cigarette smoke solution was prepared as described previously (10). In brief, cigarette smoke was withdrawn into a polypropylene syringe (35 ml) at a rate of one puff/min (10 times) and bubbled slowly into 20 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 epithelial carcinoma cell line, were grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). These cells were cultured in 10-cm dishes in the presence of 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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in 4% paraformaldehyde, rehydrated in 0.5 M citrate (SSC), and then acetylated in triethanolamine and 10 mM β-mercaptoethanol at 55°C for 2 h and then in 0.5× SSC at room temperature for 20 min. Specimens were dehydrated, air-dried, and covered with Kodak nitro blue tetrazolium nuclear track emulsion (Eastman Kodak, Rochester, NY) for autoradiography. After exposure for 7–21 days at 4°C, the slides were developed, fixed, and counterstained with hematoxylin.

**Immunoblotting for activated EGFR.** Cells were serum starved for 24 h and then stimulated with cigarette smoke solution or with transforming growth factor-α (TGF-α) for 15 min. After stimulation, cells were lysed with lysis buffer (20 mM sodium phosphate, pH 7.8, 150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 1% Triton X-100, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aproatin) and incubated for 30 min at 4°C. To remove insoluble materials, cell lysates were centrifuged at 14,000 rpm for 5 min at 4°C. Aliquots of supernatants containing equal amounts of protein were suspended in SDS sample buffer and boiled for 5 min. The resulting gel was equilibrated in the transfer buffer: 25 mM HCl, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.3. The proteins were then transferred electrophoretically to nitrocellulose membranes, which were incubated with 5% nonfat-free skimmed milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 1 h and then incubated with anti-phospho-specific EGFR monoclonal antibodies (clone 451, 1:100; NeoMarkers, Fremont, CA) that were then incubated with 50 μl of MUC5AC monoclonal antibodies (clone 451, 1:100; NeoMarkers, Fremont, CA) that were 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-goat anti-mouse IgG conjugate (1:10,000) were dispensed into each well. After 1 h, the wells were washed three times with PBS. Color reaction was developed with 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.

**In Vivo Studies**

**Drugs.** A stock solution of BIBX1522 was prepared using Solutol HS 15 (polyethylene-660-hydroxyoctearate; Badische Anilin und Soda Fabrik) as a solvent. This solution was evaporated to dryness. The residue was redissolved in 0.3 ml of methanol and once again evaporated to dryness. This stock preparation was stored at 4°C for 5 days. The solution for intratracheal installation was made up fresh each day by dissolving the stock preparation in 3 ml of prewarmed saline (40°C) to achieve final concentrations of 0.1 and 0.3%.

**Induction of goblet cell metaplasia by cigarette smoke exposure.** Male Sprague-Dawley rats weighing 250–300 g were used for the study. The animals were housed in a temperature- and humidity-controlled room and had free access to water and standard laboratory food. Animals were assigned at random to the nonsmoking control group or to the smoke-exposed control and treatment groups. Rats in the smoking groups were exposed to eight regular, nonfilter cigarettes (1.2 mg nicotine, 12 mg condensate) a day for 5–20 days.

On each day of exposure, 12 animals were placed individually in wire-mesh cages inside a Plexiglas cabinet (40 × 50 × 20 cm). Cigarette smoke was delivered into the cabinet by passing air at a flow rate of 0.3 ml/s through a burning cigarette in a chamber. The combustion time of the cigarette was ~3 min. A ventilator inside the cabinet ensured a rapid and equal distribution of the smoke. Fresh air at a flow rate of 2 l/min was delivered into the cabinet to remove the smoke. At intervals of 30 min, the smoke of a new cigarette was conducted into the cabinet. During the exposure time of 4 h, no animal, including control animals, received food or water. At other times, the animals had free access to food and water. **Inhibition of cigarette smoke-induced goblet cell metaplasia by the EGFR kinase inhibitor BIBX1522.** To evaluate the effect of EGFR kinase inhibitor on goblet cell metaplasia and mucus production, the animals were treated once daily with...
vehicle or with BIBX1522 at doses of 1 or 3 mg/kg intratra-
chally 1 h before the exposure to cigarette smoke. Treat-
ment of the animals with vehicle or BIBX1522 started on day 1
and was continued for 5 days during exposure to cigarette
smoke. The intratracheal instillation in a volume of 1 ml/kg
was performed under isoflurane anesthesia.

RNA isolation and quantification. Eight hours after the
last exposure to cigarette smoke, the animals were eutha-
nized with pentobarbital sodium. The trachea and right
main stem bronchi were removed and processed for total RNA
isolation using a Qiagen RNeasy kit according to the manu-
facturer's instructions. For RNA quantification, the real-time
PCR technology (TaqMan-PCR, ABI Prism 7700 Sequence
Detection System; PerkinElmer Applied Biosystems, Foster
City, CA) was employed. This technology has been described
in detail elsewhere (11). Briefly, during PCR cycles, the
5'-fluorescent-labeled nucleotide is released from the probe
by exonuclease activity of the TaqPolymerase; the emission
of fluorescence is detected via laser, and during proceeding
PCR cycles, an increasing fluorescence above background is
measured and documented. The signal is normalized in rela-
tion to an internal reference signal, and the software sets the
threshold cycle when the difference to the reference signal is
more than 10-fold of standard deviation. The threshold cycle
value is used for quantification of the input target number.

Primers and probes for rat MUC5AC were designed using
the PrimerExpress 1.0 program provided by PerkinElmer.
The following sequences were used for the quantification of
the rat MUC5AC: forward primer 5'-TGG GAA CCA TCA
TCT ACA ACC A-3', reverse primer 5'-TCC TGA CTA ACC
CCT TGG ACC A-3', and the FAM reporter dye-labeled
hybridization probe 5'-CCT TGA CCG CCA CTT TTA CTA
TGC GAT GT-3'.

Primers and probe for ribosomal RNA were purchased from
Biosystems Deutschland [TaqMan Ribosomal RNA
Control Reagents (VIC Probe), Patent No. 4508329]. RT-PCR
and TaqMan PCR were performed in a one-step RT-PCR
using the TaqMan EZ RT-PCR Core Reagents; forward
primer 50 nM, reverse primer 300 nM, probe 100 nM, man-
ganese acetate 2.5 mM; total RNA 5–10 ng; enzymes, reac-
tion buffer and nucleotides according to the manufactur-
er's protocol (TaqMan EZ RT-PCR Kit, PerkinElmer P/N
402877 Rev. A, 1996). Cycles were 10 min at 50°C, 30 min at
60°C, 5 min at 95°C, 40 times 20 s at 94°C, and 1 min at 59°C.
To quantify the mRNA expression, the target gene was first
normalized to the ribosomal RNA as internal standard. The
data were then expressed as the relative amount of MUC5AC
compared with a standard control tissue.

Tissue preparation and quantification of goblet cell produc-
tion. The lungs were dissected and fixed in 7% buffered
Formalin and embedded in paraffin. The left main stem
bronchus was used for immunohistochemical staining. Lung
sections were cut to include the full length of the main
intrapulmonary airway and stained sequentially with hema-
toxylin and eosin or with Alcian blue (AB)-periodic acid-
Schiff (PAS) to evaluate the total epithelial area and the area
stained for intracellular mucus glycoconjugates, respec-
tively. Goblet cell production was determined by the volume
density of AB-PAS-stained mucus glycoconjugates on the
epithelial mucosal surface using an image analysis system
(Soft Imaging System, Münster, Germany). The AB-PAS-
positive stained area, the number of goblet cells, and the total
epithelial area were measured over a length of 2 mm of the
basal lamina. The stained areas are expressed as the per-
centage of the total area stained by AB-PAS.

Tissue preparation. Surgical specimens were fixed with 4%
paraformaldehyde for 1 h and then placed in 30% sucrose for
cryoprotection overnight. The specimens were embedded in
optimal cutting temperature compound and cut as 4-μm-
thick sections.

Immunohistochemical analysis of EGFR. Immunohisto-
chemistry was performed using frozen sections. Sections
were fixed with 4% paraformaldehyde for 5 min. PBS
containing 0.05% Tween 20, 2% normal goat serum, and
Levamisol (2 mM) was used as diluent for the antibodies.
The sections were incubated with mouse monoclonal antibody
to EGFR (1:200; Calbiochem, San Diego, CA) for 2 h at
room temperature and then washed three times with PBS to
remove excess primary antibody. The sections were then incu-
bated with biotinylated horse anti-mouse IgG (Vector Lab-
oratories) at 1:200 dilution for 1 h at room temperature.
Bound antibody was visualized according to standard proto-
cols for the avidin-biotin-alkaline phosphatase complex
method. All immunohistochemical staining included control
sections unexposed to primary antibody, with substitution of
an unrelated antibody of the same isotype or preincubation
of the antibody with a 10-fold excess of immunizing peptide.
A rabbit polyclonal antibody to EGFR (1:100, Calbiochem)
also was used to confirm the staining pattern and to perform
quenching using EGFR peptide antigen, which corresponds
to amino acid residues 1005–1016 of the human EGFR (Cal-
biochem).

Statistics

All data are expressed as means ± SE. One-way analysis
of variance (ANOVA) was used to determine statistically
significant differences between groups. Scheffe’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

In Vitro Studies in NCI-H292 Cells

Cigarette smoke upregulates EGFR mRNA expres-
sion. In the control condition, NCI-H292 cells ex-
pressed EGFR mRNA constitutively. Addition of ciga-
rette smoke solution to the cells upregulated EGFR
mRNA expression within 6 h, an effect that was
increased at 12 h. Tumor necrosis factor-α (TNF-α,
used as control) also increased EGFR mRNA expres-
sion. The sense probe of EGFR showed no expression
(Fig. 1).

Cigarette smoke activates EGFR tyrosine phosphory-
lation. Because the activation of EGFR by its ligands
leads to MUC5AC synthesis (28), we examined the
effect of cigarette smoke solution on activation of
EGFR tyrosine kinase. As a positive control, we used the
EGFR ligand TGF-α, which increased EGFR-spe-
cific tyrosine phosphorylation in NCI-H292 cells (Fig.
2), as described previously (28). Similarly, cigarette
smoke solution increased EGFR-specific tyrosine phos-
phorylation but to a lesser extent (Fig. 2). Pretreat-
ment of NCI-H292 cells with BIBX1522 inhibited
EGFR tyrosine phosphorylation induced by cigarette
smoke solution and by TGF-α (Fig. 2).

Cigarette smoke increases MUC5AC expression.
Resting NCI-H292 cells showed little expression of
MUC5AC mRNA at 12 h. Addition of cigarette smoke
solution to the cells upregulated MUC5AC mRNA expression within 6 h (data not shown), an effect that was increased at 12 h (Fig. 3). TGF-α (used as control) also increased MUC5AC mRNA expression. The sense probe of MUC5AC showed no expression. Similarly, cigarette smoke solution increased MUC5AC protein synthesis within 24 h, an effect that occurred in a dose-dependent fashion (Fig. 4).

**EGFR tyrosine kinase inhibitors prevent MUC5AC gene and protein expression in NCI-H292 cells.** To test whether the cigarette smoke-induced MUC5AC gene and protein expression occurred by activation of EGFR, cells were incubated with various tyrosine kinase inhibitors. Pretreatment of the cells with selective EGFR tyrosine kinase inhibitors (BIBX1522, AG-1478) prevented MUC5AC mRNA expression (Fig. 3) and MUC5AC protein synthesis induced by cigarette smoke solution (Fig. 4). A selective tyrosine kinase inhibitor of platelet-derived growth factor (AG-1295) and a negative control for tyrphostins (A1) were without effect (Fig. 4). Furthermore, cigarette smoke-induced MUC5AC synthesis was inhibited significantly by pretreatment with a free radical scavenger (DMSO) and by SOD. These results indicate that activation of EGFR tyrosine kinase induces MUC5AC gene and protein expression in NCI-H292 cells and that oxidative stress induced by cigarette smoke is involved, at least in part, in cigarette smoke-induced MUC5AC production.

**In Vivo Studies in Rats**

Cigarette smoke increases goblet cell production in pathogen-free rats. In control animals, the airway epithelium contained sparse staining with AB-PAS (Figs. 5 and 6A). Inhalation of cigarette smoke (8 cigarettes/day for 5 days) resulted in a markedly increased area of AB-PAS staining (Figs. 5 and 6B). Inhalation of cigarette smoke also increased the number of goblet cells from 40 ± 19 to 167 ± 19 cells/mm of epithelium (P < 0.001). Inhalation of cigarette smoke also increased MUC5AC mucin gene expression (Fig. 7).

**EGFR tyrosine kinase inhibitor (BIBX1522) prevents cigarette smoke-induced goblet cell production in pathogen-free rats.** When rats were treated with BIBX1522 during cigarette smoking, the increase in AB-PAS staining was inhibited dose dependently (Figs. 5 and 6C), and the cigarette smoke-induced increase in the number of goblet cells was also prevented by BIBX1522 (3 mg/kg intratracheally). In BIBX1522-treated rats given cigarette smoke, there were 51 ± 19 cells/mm epithelium; P > 0.05 compared with control. BIBX1522 also prevented the cigarette smoke-induced MUC5AC gene expression (Fig. 7).
DISCUSSION

In the present study, we have addressed the question of whether cigarette smoke increases mucin MUC5AC synthesis via EGFR activation in airway epithelial cells. Our results show that exposure of airway epithelial cells to cigarette smoke upregulates EGFR expression and activates EGFR tyrosine phosphorylation, causing mucin MUC5AC synthesis in NCI-H292 cells. Pretreatment with selective EGFR tyrosine kinase inhibitors prevented cigarette smoke-induced EGFR tyrosine phosphorylation and MUC5AC synthesis in vitro; a non-EGFR tyrosine kinase inhibitor, a selective platelet-derived growth factor receptor kinase inhibitor (AG-1295), and a negative control (tyrphostin A1) were without effect, implicating EGFR tyrosine phosphorylation as the signaling pathway of MUC5AC synthesis induced by cigarette smoke. Most importantly, the present studies show for the first time that an EGFR tyrosine kinase inhibitor prevents cigarette smoke-induced MUC5AC synthesis in vivo and in vitro.

The mechanism by which cigarette smoke induces EGFR activation requires discussion. To induce EGFR activation, two different pathways have been reported. First, the binding of its ligands to EGFR activates the intrinsic receptor tyrosine kinase and induces tyrosine phosphorylation (15). Second, tyrosine phosphorylation of EGFR can be activated by a ligand-independent mechanism ("transactivation"). EGFR transactivation is known to occur with various stimuli such as oxidative H$_2$O$_2$ (12, 13), ultraviolet light (27), and osmotic stress (27), and with stimulation of G protein-coupled receptors by endothelin 1, lysophosphatidic acid, and thrombin (5), M$_1$ muscarinic acetylcholine receptor (31) and growth hormone (32). In our experiments, antioxidants (DMSO and superoxide dismutase (SOD)) inhibited MUC5AC protein production induced by cigarette smoke partially [n = 5, †P < 0.05, ††P < 0.01, significantly different from response to cigarette smoke (10 puffs) alone].
synthesis via the EGFR-mitogen-activated protein kinase-p44/42 mitogen-activated protein kinase pathway in vitro (29). Interestingly, cigarette smoke also is known to cause neutrophil migration into the airways (22). Thus MUC5AC synthesis in vivo might be caused both via the direct stimulation of cigarette smoke and via indirect stimulation resulting from neutrophil recruitment, activation, and release of oxygen free radicals induced by inhalation of cigarette smoke. Although ~50% of cigarette smoke-induced EGFR activation and mucin synthesis was explained by oxidative stress, the remainder of the EGFR activation and subsequent mucin synthesis induced by cigarette smoke is unknown. Because cigarette smoke-induced goblet cell metaplasia in rats has been reported to be prevented by pretreatment with an inhibitor of cyclooxygenase products, indomethacin (25), the products of the arachidonate cascade might be involved in cigarette smoke-induced EGFR activation. Indeed, a link between EGFR activation and the arachidonate cascade has been reported. Arachidonic acid induces EGFR tyrosine phosphorylation and its association with Shc, resulting in mitogen-activated protein kinase activation in epithelial cells of rabbit renal proximal tubules (9). In addition, acrolein, a product of cigarette smoke, is reported to induce MUC5AC expression in rat airways (2). Interestingly, acrolein also is reported to produce cyclooxygenase products (prostaglandins E2 and F2α) in airway epithelial cells (8, 16). Thus the inhibitory effect of indomethacin on cigarette smoke-induced goblet cell metaplasia may be due to the prostaglandin synthesis induced by acrolein. Taken together, cigarette smoke-induced EGFR activation and subsequent mucin synthesis in airways could be caused in part by the cyclooxygenase products that are upregulated by cigarette smoke. Further studies are required to determine the effects of various components of cigarette smoke (e.g., nicotine, tar, acrolein). Another factor possibly involved in cigarette smoke-induced EGFR activation is the upregulation of EGFR ligands. Oxidative stress has been shown to increase gene expression of the EGFR ligands, heparin-binding EGF-like growth factor and amphiregulin, in rat gastric epithelial cells (21). Thus it is possible that cigarette smoke, by upregulating EGFR ligands, could activate EGFR to induce mucin synthesis. Another mechanism by which cigarette smoke may enhance MUC5AC synthesis is upregulation of EGFR expression. In the present study, we found that cigarette smoke upregulated EGFR mRNA expression in NCI-H292 cells and that strong EGFR immunoreactiv-
ity was observed in airway goblet cells in patients with COPD. Similarly, a previous study showed that strong EGFR immunoreactivity was found in the bronchial epithelium of smokers (17). Thus cigarette smoke-induced goblet-cell production may involve both upregulation of EGFR expression and subsequent EGFR activation in COPD.

Regardless of the multiple mechanisms in addition to oxidative stress potentially involved, the fact that cigarette smoke-induced mucin synthesis was blocked completely by EGFR tyrosine kinase inhibitors indicates that EGFR activation has a principal role in cigarette smoke-induced MUC5AC synthesis.

In humans, we suggest that the EGFR cascade in glands could be important in the exaggerated mucus production in conducting airways in chronic bronchitis (1) in which the excessive sputum is believed to be derived largely from hyperplastic glands (30). Furthermore, prolonged cigarette smoking has been suggested to be associated with pathological changes in peripheral airways, including goblet cell hyperplasia (4). These results implicate cigarette smoke as a regulator of epithelial cell differentiation that may result in abnormal induction of mucus-producing cells in airways. Most importantly, inhibition of EGFR activation is proposed as therapy in hypersecretory airway diseases. Proof of the concept in humans will require testing of patients with these diseases.

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