Niflumic Acid and AG-1478 Reduce Cigarette Smoke-Induced Mucin Synthesis*

The Role of hCLCA1

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Background: **Cigarette smoke induces bronchial mucus secretion. However, the mechanism of this induction is still unidentified. In this study, we investigated the role of the putative calcium-activated chloride channel 1 (CLCA1) and its blocker, niflumic acid, in cigarette smoke-induced mucin synthesis both *in vivo* and *in vitro*.**

Methods and results: **Sprague-Dawley rats were exposed to cigarette smoke for 4 weeks. The CLCA1, epidermal growth factor receptor (EGFR), and MUC5AC expressions were increased in the trachea and lung tissues. Goblet-cell hyperplasia with marked mucin staining was detected in the tracheal and bronchial epithelium. In the human bronchial epithelial cell line NCI-H292, cigarette smoke solution also induced mucin production as well as the RNA and protein expressions of CLCA1, EGFR, and MUC5AC. Both *in vivo* and *in vitro*, the induction of MUC5AC and mucin synthesis were inhibited by niflumic acid, and/or a selective EGFR tyrosine kinase inhibitor, AG-1478. Niflumic acid also blocked the epidermal growth factor-induced MUC5AC and mucin staining in the NCI-H292 cell line.**

Conclusion: **Both EGFR and niflumic acid-sensitive chloride channels (probably CLCA1) are dependently affecting the mucin production as a part of a single complex signaling pathway. CLCA1 may be a key signaling member that can be targeted with pharmacologic interventions to treat mucus hypersecretion.** ***(CHEST 2007; 131:1149–1156)***

Key words: calcium-activated chloride channel; COPD; epidermal growth factor receptor; MUC5AC

Abbreviations: CLCA = calcium-activated chloride channel; CLCA1 = calcium-activated chloride channel 1; DMSO = dimethyl sulfoxide; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; hCLCA1 = human calcium-activated chloride channel 1; IL = interleukin; mCLCA3 = murine calcium-activated chloride channel 3; mRNA = messenger RNA; PAS = periodic acid-Schiff; pEGFR = anti-phosphospecific epidermal growth factor receptor; Th2 = T-helper type 2; TNF = tumor necrosis factor

Chronic bronchitis is a clinical syndrome characterized by chronic sputum production and is a major phenotype of COPD. Cigarette smoking is the main risk factor for COPD. Although several studies[1] have shown that cigarette smoke can induce mucus production, the mechanism of this induction remains unexplained. MUC5AC is the major respiratory mucin in goblet-cell secretion.[2] Mucin synthesis in response to various stimuli is regulated by the epidermal growth factor receptor (EGFR) system.[3]

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Cigarette smoke causes activation of EGFR via phosphorylation of tyrosine residues that leads to extracellular signal-regulated kinase activation that is required for activator protein 1-containing response element to bind JunD and Fra-2 causing upregulation of MUC5AC at both messenger RNA (mRNA) and protein levels.[4]

Human calcium-activated chloride channel 1 (hCLCA1) has been shown to be critical for airway mucus production. The expression of hCLCA1 is strongly induced in the airway goblet cells in human asthmatics[5,6] and COPD patients.[7] In addition, transfection of the hCLCA1 gene into the human mucoepidermoid cell line NCI-H292 induces mucin production.[8] Its murine counter part, murine calcium-activated chloride channel 3 (mCLCA3), was the most highly induced gene at all time points in the asbestos-induced mucus production,[9] and was markedly induced with MUC5AC in respiratory syncytial virus-infected, allergically sensitized mice.[10] Furthermore, some polymorphisms and haplotypes within the hCLCA1 gene have been shown to be associated with bronchial asthma[11] and COPD.[12] Taken together, these data indicate that calcium-activated chloride channel 1 (CLCA1) is involved in several pulmonary diseases that are characterized by increased mucus production.

Materials and Methods

In Vivo *Studies*

Animals and Exposure to Cigarette Smoke: Male Sprague-Dawley rats weighing 200 to 250 g were randomly assigned to the nonsmoking group (n = 4), the smoke-exposed group (n = 20; 4 animals killed at 2, 7, 14, 21, and 28 days), or the smoke-exposed treatment group (n = 12; 4 animals for each treatment protocol). Rats were exposed to the whole smoke from six nonfiltered cigarettes per day, 5 d/wk, for 2 to 28 days by the whole-body exposure method at the rate of one puff per minute. After the exposure of one cigarette smoke, animals were allowed to breathe fresh air for 20 min before the next cigarette smoke was administered.

Treatment With Niflumic Acid and/or AG-1478: Niflumic acid (Sigma-Aldrich; St. Louis, MO) is a blocker of calcium-activated chloride channels (CLCAs). AG-1478 (LC Laboratories; Woburn, MA) is a selective EGFR tyrosine kinase inhibitor. To examine the effect of inhibiting CLCA1 and/or EGFR on the cigarette smoke-induced mucus production, the animals in the smoke-exposed treatment group were injected intraperitoneally 30 min/d before the smoke exposure with either vehicle, niflumic acid (3 mg/kg), AG-1478 (3 mg/kg), or both niflumic acid and AG-1478.

RNA Isolation and Quantification of Gene Expressions: Two hours after the last smoke exposure, the animals were killed; RNA was then isolated from the trachea and lungs. RNA expression measurement was done using the real-time TaqMan-polymerase chain reaction technology (ABI Prism 7000 Sequence Detection System; Applied Biosystems; Foster City, CA); the quantification of CLCA1 and EGFR was done using the Assays-on-Demand gene expression assay primer/probe. The quantification of MUC5AC was done using the following sequences: probe 5′-[FAM]-CTGTCCATTCACGTGCC-[MGB]-3′, sense primer 5′-TGGAGAAGCCATACCAACAACA-3′ and antisense primer 5′-CAAGGCTGGTATACTTGGTTTTCA-3′. The TaqMan 18s recombinant RNA primer/probe mixture (Applied Biosystems) was used as a housekeeping gene to normalize for differences in the amounts of mRNAs. Samples were run simultaneously in triplicate and repeated three times.

Western Blotting: Protein was separated from lung tissues, and its concentration was measured by standard techniques. The samples were separated on polyacrylamide gel, and then electrotransferred onto Immun-Blot polyvinylidene fluoride membrane (Bio-Rad; Hercules, CA). Membranes were blocked in skimmed milk and then probed with the following primary polyclonal antibodies (dilute 1:200 to approximately 1,000): a rabbit anti-Gob-5 (kindly provided by Takeda Pharmaceutical; Tsukuba, Japan), a rabbit anti-EGFR, a goat anti-phosphospecific (Tyr 1173) EGFR (pEGFR), and a goat anti-glyceraldehyde-3-phosphate-dehydrogenase antibodies (Santa Cruze Biotechnology; Santa Cruz, CA). The membranes were subsequently incubated in horseradish peroxidase-coupled secondary antibodies. Band formation was visualized by simple staining with the DAB substrate. The bands were quantified by densitometric scanning using ImageJ (available at: http://rsb.info.nih.gov/ij/index.html). Data are expressed as percentage of control, and the expression of the different proteins were normalized to glyceraldehyde 3-phosphate dehydrogenase readings.

Morphometric Analysis: Paraffin-embedded lungs with small portions of the tracheas were sectioned transversely and stained with hematoxylin-eosin or with periodic acid-Schiff (PAS) to evaluate the total epithelial area and the area stained for intracellular mucin, respectively. These areas were measured using a digital camera (FX380; Olympus; Tokyo, Japan). Eight different images, four from the trachea and four from major bronchi, were analyzed for each rat. Data are expressed as the percentage of PAS-stained area to the total epithelial area.

In Vitro *Studies*

Smoke Solution Preparation: Smoke solution was prepared as previously described[13] with some modifications. In brief, one cigarette and a 50-mL syringe were attached to a three-way stopcock. Then 1, 4, or 10 puffs of smoke were repeatedly withdrawn to the syringe and bubbled into 20 mL of serum-free RPMI 1640 medium containing 25 mmol/L hydroxylethyl piperazine-ethanesulfonic acid buffer. The smoke solution was filtered and used immediately.

Cell Culture and Exposure to Smoke Solution: The human bronchial mucoepidermoid carcinoma cell line NCI-H292 (American Type Culture Collection; Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2. The cells were cultured in 100-mm culture dishes for RNA studies and in Lab-Tek eight-chamber slides (Nalge Nunc International; Naperville, IL) for PAS staining studies. At confluence, the medium was changed into serum-free RPMI 1640 and the cells were cultured for 24 h more. Next, the cells were incubated in the smoke solution for 1 h. The cells were then washed and cultured with serum-free medium for 1, 18, 24, or 36 h. In treatment studies, the culture medium was supplemented with niflumic acid (300 μmol/L), AG-1478 (10 μmol/L), or both from 1 h before changing the medium into the smoke solution to the end of the culture. As controls, cells were incubated with serum-free medium alone or supplemented with the same drugs for the same duration. Since dimethyl sulfoxide (DMSO) was

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