

Niflumic acid and MSI-2216 reduce TNF- α -induced mucin expression in human airway mucosa

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Background: Human chloride channel, calcium-activated 1 (hCLCA1) has been shown to induce mucin (MUC) gene expression and mucus production in bronchial epithelial cells. **Objective:** To investigate whether blocking hCLCA1 decreases mucus production.

Methods: Expression of hCLCA1 and mucus was stimulated with TNF- α in human upper airway mucosal explant tissue. MUC5AC mRNA and mucus protein expression was blocked by inhibiting hCLCA1 by using channel blockers (niflumic acid [NFA] and MSI-2216) without and with TNF- α stimulation. Expression of MUC5AC, hCLCA1, and COX-2 mRNA was quantified by using real-time PCR. Mucus protein was assessed by periodic acid Schiff staining. Laser capture microdissection was performed to quantify hCLCA1 and MUC5AC mRNA expression in epithelial cells derived from mucosal explant tissue. **Results:** TNF- α significantly increased MUC5AC and hCLCA1 mRNA as well as mucus and hCLCA1 protein expression in the mucosal explant tissue ($P < .05$). Inhibition of hCLCA1 with NFA or MSI-2216 showed a significant dose-dependent reduction of mucus production for both blockers in the mucosal explant tissue ($P < .05$). MUC5AC mRNA was also decreased by both blockers in the whole mucosal tissue and in laser-captured mucosa epithelial cells.

Conclusions: Unstimulated and TNF- α -induced mucin expression could be decreased by NFA and MSI-2216. Inhibiting hCLCA1 may be a potential new approach to reduce mucus overproduction. (J Allergy Clin Immunol 2005;115:266-71.)

Key words: Airway, explant model, hCLCA1, inhibition, laser capture microdissection, MSI-2216, MUC5AC, mucin, niflumic acid, TNF- α

Mucus hypersecretion is a common feature of chronic inflammatory airway diseases such as bronchial asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF).¹⁻³ It has been suggested that this might occur via human chloride channel, calcium-activated 1 (hCLCA1). Introduction of hCLCA1 into the human mucocypidermoid cell line NCI-H292 induced mucin (MUC) gene expression and mucus production.⁴ Previous studies found increased expression of hCLCA1 in the airways in bronchial asthma,⁵ CF,⁶ and COPD (H.P. Hauber, unpublished data, November 2003), further supporting the role of this channel in mucus hypersecretion.

Inhibition of hCLCA1 *in vitro* (NCI-H292 cells) and its murine orthologue mCLCA3 *in vivo* by the chloride channel blocker niflumic acid (NFA) reduced MUC5AC gene expression and mucus overproduction.⁷ However, the effect of NFA on mucin expression in human airway mucosa has not been determined yet. Therefore, we sought to inhibit hCLCA1 by using channel blockers and to study the effect on MUC gene expression and mucus production in TNF- α -stimulated and unstimulated tissue. We found that TNF- α stimulates hCLCA1 and mucus expression in an *ex vivo* model of human upper airway mucosa, and chloride channel blockers inhibit mucin expression. We hypothesized that inhibition of hCLCA1 would decrease MUC gene expression and mucus production.

METHODS

Tissue culture

Upper airway mucosa (sinus) specimens were obtained from patients with chronic sinusitis undergoing sinus surgery who had given informed consent before the procedure. Serial sections (5 mm thick) of tissue were placed on 0.4- μ m well inserts (Millipore, Bedford, Mass) in 2 mL defined medium^{8,9} and incubated in 5% CO₂/95% air. Tissue was stimulated for 24 hours as described here. The maximum delay between removal of tissue and culture was 1 hour. After culture, tissue was cut into 2 parts. One part was fixed in 4%

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Abbreviations used

ALAS-1:	Δ -aminolevulinate synthase 1
CF:	Cystic fibrosis
COPD:	Chronic obstructive pulmonary disease
EF-1 α :	Elongation factor 1 α
hCLCA1:	Human chloride channel, calcium-activated 1
HKG:	Housekeeping gene
LCM:	Laser capture microdissection
MUC:	Mucin
NFA:	Niflumic acid
PAS:	Periodic acid–Schiff
QRTPCR:	Quantitative real-time PCR
rhTNF- α :	Recombinant human TNF- α

paraformaldehyde, washed in a solution of 15% sucrose/PBS, and blocked in OCT medium by snap-freezing in isopentane cooled in liquid nitrogen. The other part was put into RNA stabilizing solution (RNAlater; Ambion, Austin, Tex) for RNA extraction.

Sections used for laser capture microdissection (LCM) were immediately blocked in OCT medium by snap-freezing in isopentane cooled in liquid nitrogen.

Blocking hCLCA1 function in unstimulated and in TNF- α -stimulated mucosal explant tissue

To inhibit hCLCA1 function, specific blockers (NFA and MSI-2216, an NFA analogue; Genaera Corp) were used. Tissue samples were cultured without ($n = 6$) and with recombinant human (rh)TNF- α (10 ng/mL; R&D Systems, Minneapolis, Minn; $n = 9$; Fig 1) with and without either NFA (10, 100, and 500 μ mol/L) or MSI-2216 (5, 50, 100 μ mol/L) for 24 hours. Experiments in Fig 2, A and B, used samples different from Fig 2, C and D, because it was not feasible to cut the small tissue into 14 pieces for 14 different culture conditions. In 292 cells overexpressing hCLCA1, MSI-2216 is significantly more potent than NFA in inhibiting mucin production (H.L. Hung, unpublished data, January 2004). Blockers were applied 1 hour before stimulation with TNF- α to ensure binding to hCLCA1 protein.

Immunohistochemistry

Tissue sections (7 μ m) were stained with periodic acid–Schiff (PAS) to identify mucus glycoconjugates and mucus-producing goblet cells. Immunostaining was performed with the previously described alkaline phosphatase–antialkaline phosphatase technique¹⁰ to assess hCLCA1 protein (anti-hCLCA1 antibody; Genaera Corp), T lymphocytes (anti-CD3 antibody; DAKO, Mississauga, Ontario, Canada), macrophages (anti-CD68 antibody; DAKO), neutrophils (antineutrophil elastase antibody; DAKO), and eosinophils (anti-major basic protein antibody; kindly provided by Dr Moqbel).

Tissue preparation for LCM

Tissue samples ($n = 3$) were incubated for 24 hours in the presence of rhTNF- α (10 ng/mL), rhTNF- α + NFA (100 μ mol/L), and TNF- α + MSI-2216 (50 μ mol/L) for LCM. For LCM, 5- μ m-thick sections were cut from frozen tissue blocked in OCT medium as described. The frozen sections were placed on plain glass slides (Fisher Scientific, Pittsburgh, Pa) and then immediately fixed in 75% ethanol for 30 seconds, followed by rehydration in ribonuclease-free deionized H₂O (30 seconds), staining with hematoxylin (15 seconds), washing in RNase-free deionized H₂O (30 seconds), and dehydration

through 75%, 95%, and 100% RNase-free ethanol (30 seconds each). After cleaning in xylene for 3 minutes, the slides were air-dried for 5 minutes before LCM was performed.

Tissue sampling by LCM

Slides were completely air-dried and kept desiccated to prevent the activation of endogenous RNase in the tissues. A minimum of 8 sections of each condition was prepared for LCM. LCM of surface epithelial cells was performed by using the PixCell II System (Arcturus Engineering, Mountain View, Calif) with the following parameters: laser diameter, 15 μ m; pulse duration, 1.5 ms; and amplitudes, 80 to 90 mW. The whole epithelium from each section was retrieved. A total of 12,000 to 15,000 bursts of the laser was used to collect the cells from each stimulation condition. The interval between staining the slides and completing microdissection was 1 to 2 hours. After samples were captured on transfer film (Cap-Sure Macro LCM Caps, Arcturus Engineering), cells were lysed with RLT lysis buffer (Qiagen, Mississauga, Ontario, Canada). Lysed cell samples were stored at -20°C until RNA extraction.

RNA extraction and reverse transcription

Whole tissue samples were homogenized before RNA extraction. RNA from whole tissue samples and LCM-captured epithelial cells was extracted by using an RNeasy Mini Kit (Qiagen). Reverse transcription was performed with 0.5 to 1.0 μ g RNA per reaction by using Superscript II reverse transcriptase (200 U per reaction; Invitrogen, Burlington, Ontario, Canada) in the presence of an RNase inhibitor (RNase Out, Invitrogen). For LCM-derived RNA, the concentration of reverse transcriptase was adjusted to 50 U per reaction because of the lower amount of RNA (50–100 ng per reaction). The RNA was reverse transcribed in 30 μ L total volume at 65°C for 10 minutes, at 42°C for 60 minutes, and at 100°C for 1 minute. The resultant first-strand cDNA was used as a template for PCR.

Quantitative real-time PCR

Quantitative real-time PCR (QRTPCR) was performed by using a LightCycler system (Roche Diagnostics, Mannheim, Germany). MUC5AC was selected to measure mucin gene expression because it encodes for the most prominent secreted mucin in human airways.¹¹ MUC5AC mRNA, hCLCA1 mRNA, and COX-2 mRNA expression were quantified by using QRTPCR. Δ -aminolevulinate synthase 1 (ALAS-1) was used as the housekeeping gene (HKG) for whole tissue samples and elongation factor 1 α (EF-1 α) as the HKG for LCM because the efficiency of EF-1 α primers was higher in LCM samples than that of ALAS-1 primers. However, there was no difference in the results when EF-1 α and ALAS-1 were used as HKGs in the same whole tissue sample. HKGs were used to normalize differences of reverse transcription efficiency and different amounts of mRNA being transcribed. Primers were based on published mRNA sequences for MUC5AC, hCLCA1, COX-2, ALAS-1, and EF-1 α (GenBank Data Library accession numbers AJ001402-MUC5AC, AF039400-hCLCA1, AJ634912-COX-2, NM000688-ALAS-1, XM291943-EF-1 α) and were designed to span at least 2 exons to avoid amplification of genomic DNA. This was confirmed by performing the assay without and with a DNase step for each gene. Primer sequences were as follows: MUC5AC: 5'-TGA CCC ACC AGT GTG AGA AG (forward), 5'-GGA TGA TCA GGC TCC TAT GG (reverse); hCLCA1: 5'-GCA AGG TGG CTT TGT AGT GG (forward), 5'-GGA ATT TGC TGG TGT CCT TG (reverse); COX-2: 5'-CCT TCC TCC TGT GCC TGA TG (forward), 5'-ACA ATC TCA TTT GAA TCA GGA AGC T (reverse); ALAS-1: 5'-CCC ATG AGT TTG GAG CAA TC (forward), 5'-ACC CTC CAA CAC AAC CAA AG (reverse); EF-1 α : 5'-CCA CCC TAC AGC CAG AAG AG (forward), 5'-ACG AGT TGG TAG GAT GC (reverse). Specific

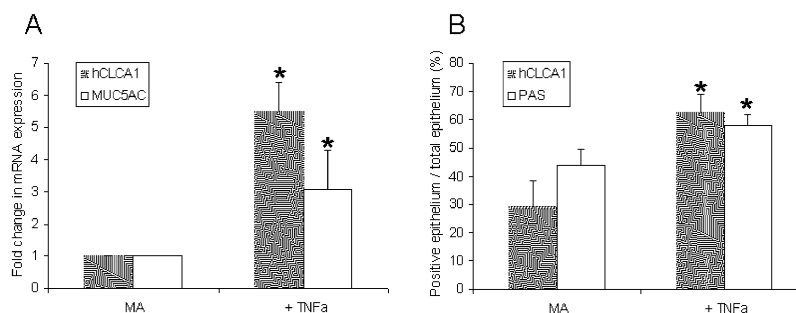


FIG 1. Stimulation with TNF- α increases expression of hCLCA1 and MUC5AC mRNA (**A**) as well as expression of hCLCA1 and mucus protein (**B**) (n = 9). Mean values $\pm$ SEMs are shown as columns. * P < .05 vs medium alone (MA).

TABLE I. Inflammatory cell profiles at baseline and after culture for 24 hours

	Baseline	Medium alone	+ TNF- α
Lymphocytes	54.9 $\pm$ 8.7	56.9 $\pm$ 12.9	44.0 $\pm$ 8.5
Macrophages	23.6 $\pm$ 3.6	22.1 $\pm$ 4.8	16.6 $\pm$ 5.4
Neutrophils	9.5 $\pm$ 2.3	6.7 $\pm$ 2.1	5.4 $\pm$ 2.8
Eosinophils	2.2 $\pm$ 4.7	11.3 $\pm$ 4.9	9.4 $\pm$ 2.8

Cell counts per square millimeter of submucosa. Mean values $\pm$ SEMs.

amplification using these primers was confirmed by ethidium bromide staining of the predicted size of the PCR products on an agarose gel. PCR was performed by using the QuantiTect SYBR Green PCR Kit (Qiagen) with the appropriate primers and samples according to the manufacturer's protocol. In brief, 1 μ L cDNA was added to 10 μ L 2X QuantiTect SYBR Green PCR master mix, 8 μ L RNase-free water, and 0.5 μ L of each primer (20 μ mol/L), resulting in a total volume of 20 μ L. All PCR experiments were performed in triplicate.

For generation of standards, mRNA from epithelial cells from cell culture (Calu-3) was reverse-transcribed into cDNA and amplified with real-time PCR by using the appropriate primers for ALAS-1, EF-1 α , MUC5AC, hCLCA1, or COX-2. Amplified transcripts were cloned into the pGEM-T vector (Promega Corp, Madison, Wis). *Escherichia coli* was transformed with the vector containing the gene of interest. The bacteria were grown and plasmid DNA was isolated. Digestion and sequence analysis was performed to ensure that the plasmid DNA was containing the gene of interest. After quantification of the amount of DNA, dilution series were performed.

Data analysis and quantification

Protein expression of PAS or hCLCA1 was expressed as percentage of positive epithelial cells per total epithelium by using surface areas. Inflammatory cells were expressed per square millimeter of submucosa. At least 2 slides were analyzed from every section. All slides were coded and counted by 2 experienced observers. The within-observer coefficient of variation for repeated measures was less than 5%. For QRT-PCR, the amount of mRNA/cDNA was determined by using standard curves constructed from serially diluted plasmids containing the PCR fragments of interest. Ratios of target gene:HKG were calculated, and values were normalized to either no stimulation or stimulation with rhTNF- α .

Statistics

An overall ANOVA, followed by multiple testing with the Bonferroni correction, was performed. Differences between con-

ditions were assessed by means of post hoc pairwise comparison with the Dunnett test. A P value less than .05 was considered statistically significant. All values are given as means $\pm$ SEMs if not otherwise stated.

RESULTS

Subject characteristics

Biopsy specimens from a total of 18 subjects were included in the study. Tissue samples from 6 patients (4 male, 2 female; 3 atopic, 3 nonatopic; median age, 47 years) were cultured without TNF- α for blocking experiments in Fig 2, A and B. Samples from 9 patients (5 male, 4 female; 2 atopic, 7 nonatopic; median age, 52 years) were cultured in the presence of TNF- α for blocking experiments in Fig 2, C and D. These samples were also used to analyze the effect of TNF- α in Fig 1. Specimens from 3 patients (1 male, 2 female; 1 atopic, 2 nonatopic; median age, 48 years) were used for LCM experiments.

Inflammatory cell profiles at baseline and after culture

Table I summarizes the inflammatory cell profiles at baseline, after 24 hours of culturing in medium alone, and after stimulation with TNF- α . No significant difference was found (P > .05). There was also no significant difference between atopic and nonatopic patients (data not shown). Neither NFA nor MSI-2216 significantly changed the numbers of inflammatory cells at concentrations used in this study (data not shown).

Induction of hCLCA1 and MUC5AC in mucosal explant tissue

Stimulation with TNF- α significantly increased hCLCA1 (5.5-fold) and MUC5AC (3-fold) mRNA expression in upper airway mucosa compared with unstimulated samples (P < .05; n = 9; Fig 1, A). hCLCA1 immunostaining and PAS staining showed significantly higher percentages of hCLCA1-expressing (62.5% $\pm$ 6.4%) and mucus-producing epithelial cells (58.2% $\pm$ 3.6%) when treated with TNF- α compared with medium alone (29.5% $\pm$ 8.7% and 39.1% $\pm$ 5.5%, respectively; P < .05; Fig 1, B). Culture itself had no significant effect

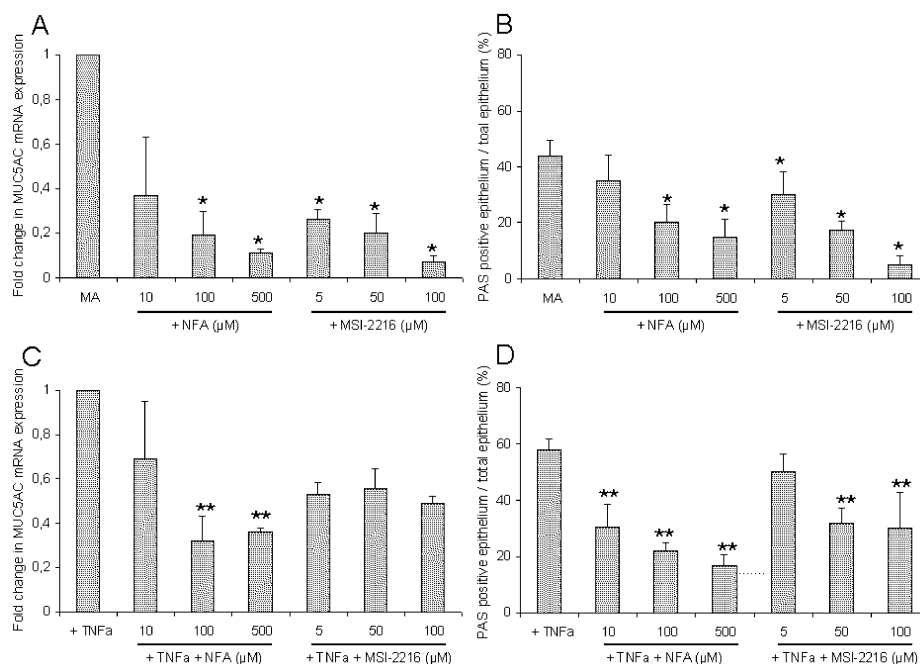


FIG 2. Effect of different concentrations of hCLCA1 blockers NFA or MSI-2216 on unstimulated (A,B) and TNF- α -stimulated (C,D) MUC5AC mRNA and mucus protein expression in human airway mucosa. Mean values $\pm$ SEMs are shown as columns. * $P < .05$ vs medium alone (MA). ** $P < .05$ vs TNF- α stimulation.

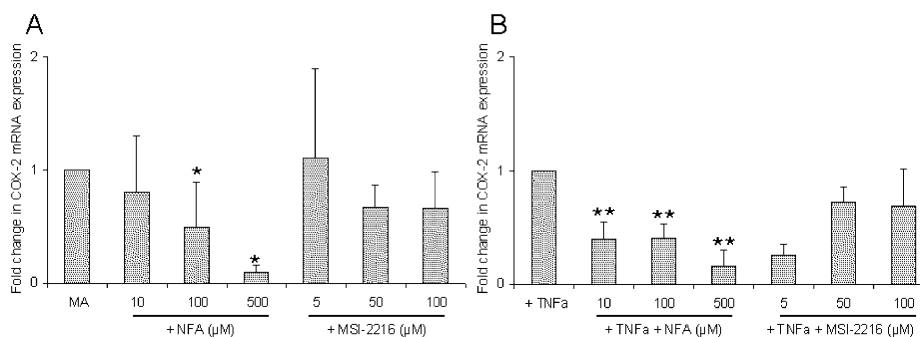


FIG 3. Effect of different concentrations of hCLCA1 blockers NFA or MSI-2216 on unstimulated (A) and TNF- α -stimulated (B) COX-2 mRNA expression in human airway mucosa. Mean values $\pm$ SEMs are shown as columns. * $P < .05$ vs medium alone (MA). ** $P < .05$ vs TNF- α stimulation.

on mucus expression (approximately 35% PAS-positive epithelial cells at baseline).

Blocking MUC5AC mRNA expression and mucus production in mucosal explant tissue

Niflumic acid significantly decreased MUC5AC mRNA and mucus protein expression at concentrations of 100 μ mol/L and 500 μ mol/L in unstimulated tissue ($P < .05$; Fig 2, A and B). MSI-2216 significantly reduced MUC5AC mRNA and mucus protein expression at all used concentrations ($P < .05$; Fig 2, A and B). In TNF- α -stimulated tissue, NFA significantly decreased mucus protein expression at all used concentrations ($P < .05$), whereas the effect on MUC5AC mRNA expression was

significant at concentrations of 100 μ mol/L and 500 μ mol/L ($P < .05$; Fig 2, C and D). MSI-2216 significantly reduced mucus protein expression at concentrations of 50 μ mol/L and 100 μ mol/L ($P < .05$). MSI-2216 partially inhibited MUC5AC RNA expression, but this inhibitory effect did not reach statistical significance ($P > .05$; Fig 2, C and D). For both blockers, a dose-dependent inhibiting effect on mucus protein expression could be observed.

Effect of TNF- α , NFA, and MSI-2216 on COX-2 expression in mucosal explant tissue

COX-2 has been shown to induce MUC5AC mRNA expression,¹² and NFA was known to inhibit the

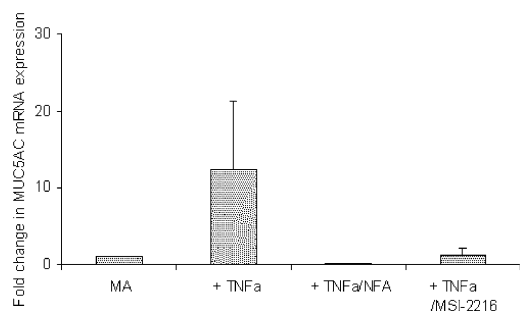


FIG 4. MUC5AC mRNA expression in LCM samples in medium alone (MA), with TNF- α , with TNF- α + NFA 100 μ mol/L, and with TNF- α + MSI-2216 50 μ mol/L ($n = 3$). Mean values $\pm$ SEMs are shown as columns.

enzymatic activity of COX-2. To determine whether NFA and MSI-2216 may inhibit mucin expression partly through inhibiting COX-2, COX-2 RNA expression was determined. TNF- α significantly increased COX-2 mRNA expression compared with medium alone (4-fold; $P < .05$; data not shown). In unstimulated tissue, COX-2 mRNA expression was significantly decreased in the presence of NFA at concentrations of 100 μ mol/L and 500 μ mol/L ($P < .05$), whereas MSI-2216 had no significant effect (Fig 3, A). NFA significantly reduced COX-2 mRNA expression at all used concentrations in TNF- α -stimulated tissue, whereas no significant effect was observed with MSI-2216 (Fig 3, B).

Stimulation and blocking of MUC5AC and hCLCA1 in laser-captured epithelial cells from mucosal explant tissue

Submucosal glands were found in most (but not all) of the sections. Glands showed PAS-positive epithelial cells. Because submucosal glands have been shown to express MUC5AC,¹³ this could influence quantification of MUC5AC mRNA expression. Therefore, LCM was performed to quantify MUC5AC mRNA expression in the surface epithelium alone. TNF- α increased MUC5AC (12-fold) and hCLCA1 (3-fold; data not shown) mRNA expression in laser-captured epithelial cells. Both NFA and MSI-2216 decreased MUC5AC mRNA expression (100-fold and 10-fold, respectively) in TNF- α -stimulated epithelial cells (Fig 4).

DISCUSSION

Excessive mucus production contributes to airway obstruction and favors bacterial infection and inflammation. Therefore, reducing mucus production could have profound benefits for these patients. In the current study, we demonstrated that MUC5AC gene and mucin expression could be reduced by hCLCA1 blocking in an *ex vivo* model of human airway mucosa.

Previous studies have shown that TNF- α can induce MUC gene expression and mucus production.¹⁴⁻¹⁶ We

chose an *ex vivo* model of human airway mucosa that is closer to an *in vivo* situation than cell line studies. In this explant model, TNF- α was added to mucosa and can also stimulate inflammatory cells. It is possible that the effects observed in the mucosa explant model were not mediated by the applied cytokine alone, but also by stimulating inflammatory cells to release other cytokines and chemokines. However, the concentration of TNF- α was higher than that normally chosen for stimulation of lymphocytes or monocytes. In addition, we did not find significant changes in the numbers of inflammatory cells after stimulation with TNF- α compared with no stimulation.

Although some reports have considered TNF- α to act mainly to preserve RNA stability of mucin genes,¹⁴ others have demonstrated direct induction of MUC5AC mRNA.¹⁶ With regard to the long duration of culture (24 hours), we cannot exclude the possibility that TNF- α also stabilized MUC5AC mRNA in our experiments. We selected a 24-hour period because time course studies with cell cultures in our laboratory showed a significant increase of mucin protein only after 18 to 24 hours in goblet cells (Calu-3; H.P. Hauber, unpublished data, July 2003).

Increased expression of TNF- α has been found in bronchial asthma, COPD, and CF.¹⁷⁻¹⁹ Our report is the first to demonstrate direct upregulation of hCLCA1 expression by TNF- α . By inhibiting hCLCA1 function in the *ex vivo* model of human airway mucosa, both blockers (NFA and MSI-2216) significantly reduced mucus protein expression in epithelial cells, similar to the effect of MSI-2216 in an animal model.²⁰ This inhibitory effect was also observed in unstimulated mucosa, suggesting that baseline hCLCA1 function was also inhibited by the blockers. Because TNF- α has been shown to induce MUC5AC mRNA expression directly in epithelial cells via extracellular-regulated kinase and p38 mitogen-activated protein kinases,¹⁶ we cannot rule out the possibility that TNF- α increased mucin expression both directly and by upregulating hCLCA1. This is consistent with the finding that TNF- α -induced mucin expression could be reduced by approximately 50% by inhibiting hCLCA1. Cell line experiments using Calu-3 cells confirmed the findings in the explanted tissue (data not shown). By performing LCM on mucosal explant tissue, we were able to investigate MUC5AC and hCLCA1 mRNA in epithelial cells without any contamination of other cell types. These data further confirmed the findings from the whole tissue explants.

Previous data have shown that NFA could also block COX-2.²¹ This enzyme has been demonstrated to induce mucin gene expression.¹² Therefore, we cannot rule out the possibility that part of the mucus blocking effect was a result of inhibition of COX-2. In our study, COX-2 mRNA expression was significantly decreased with NFA, whereas MSI-2216 had no significant effect. However, we did not examine protein levels. It is tempting to speculate that MSI-2216 is more specific in blocking hCLCA1 than NFA. This has must be examined further. NFA inhibits COX-2 enzymatic activity at much lower concentrations (approximately 100-fold) than those used in the current

study. Therefore, COX-2 function should have been totally blocked at all concentrations used in our experiments. NFA blocks hCLCA1 function at the concentrations used in this study, as demonstrated in electrophysiological experiments.²² Together, these results suggest that NFA may inhibit MUC5AC expression through both COX-2 inhibition and hCLCA1 inhibition.

In summary, the results in the current study show that unstimulated and TNF- α -induced mucus production can be reduced by hCLCA1 blocking agents in an *ex vivo* model of human airway mucosa. This is the first study to investigate the effects of hCLCA1 blockers in an *ex vivo* model on whole human airway mucosa. It is also the first report to show that cytokines other than T_H2-type cytokines can induce hCLCA1. The fact that mucin expression could be decreased in this physiologically relevant explant model suggests that blocking hCLCA1 may be a promising new approach to reduce mucus production. Further studies are warranted to examine the effect of blocking hCLCA1 in clinical studies.

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