Effects of dexamethasone on Muc5ac mucin production by primary airway goblet cells

Wenju Lu, Erik P. Lillegard, and K. Chul Kim. Effects of dexamethasone on Muc5ac mucin production by primary airway goblet cells. Am J Physiol Lung Cell Mol Physiol 288: L52–L62, 2005. First published September 10, 2004; doi:10.1152/ajplung.00104.2004.—Mucous hypersecretion associated with airway inflammation is reduced by glucocorticoids. Two mechanisms of glucocorticoid-mediated inhibition of mucus production have been proposed, direct inhibition of mucus production by airway epithelial cells and indirectly through inhibition of proinflammatory mediators that stimulate mucus production. In this study, we examined the effect of dexamethasone (DEX) on mRNA expression and synthesis of MUC5AC by A549 human lung adenocarcinoma cells as well as Muc5ac and total high-molecular-weight (HMW) mucins by primary rat tracheal surface epithelial (RTSE) cells. Our results showed that in primary RTSE cells, DEX 1 dose dependently suppressed Muc5ac mRNA levels, but the levels of cellular Muc5ac protein and HMW mucins were unaffected; 2 did not affect constitutive or UTP-stimulated mucin secretion; 3 enhanced the translation of Muc5ac; and 4 increased the stability of intracellular Muc5ac protein by a mechanism other than the inhibition of the posttranslational degradation. In A549 cells, however, DEX suppressed both MUC5AC mRNA levels and MUC5AC protein secretion in a dose-dependent manner. We conclude that whereas DEX inhibits the levels of Muc5ac mRNA in primary RTSE cells, the levels of Muc5ac protein remain unchanged as a consequence of increases in both translation and protein stability. Interestingly, some of the effects of DEX were opposite in a cell line.

Wenju Lu, Erik P. Lillegard, and K. Chul Kim. Effects of dexamethasone on Muc5ac mucin production by primary airway goblet cells. Am J Physiol Lung Cell Mol Physiol 288: L52–L62, 2005. First published September 10, 2004; doi:10.1152/ajplung.00104.2004.—Mucous hypersecretion associated with airway inflammation is reduced by glucocorticoids. Two mechanisms of glucocorticoid-mediated inhibition of mucus production have been proposed, direct inhibition of mucus production by airway epithelial cells and indirectly through inhibition of proinflammatory mediators that stimulate mucus production. In this study, we examined the effect of dexamethasone (DEX) on mRNA expression and synthesis of MUC5AC by A549 human lung adenocarcinoma cells as well as Muc5ac and total high-molecular-weight (HMW) mucins by primary rat tracheal surface epithelial (RTSE) cells. Our results showed that in primary RTSE cells, DEX 1 dose dependently suppressed Muc5ac mRNA levels, but the levels of cellular Muc5ac protein and HMW mucins were unaffected; 2 did not affect constitutive or UTP-stimulated mucin secretion; 3 enhanced the translation of Muc5ac; and 4 increased the stability of intracellular Muc5ac protein by a mechanism other than the inhibition of the posttranslational degradation. In A549 cells, however, DEX suppressed both MUC5AC mRNA levels and MUC5AC protein secretion in a dose-dependent manner. We conclude that whereas DEX inhibits the levels of Muc5ac mRNA in primary RTSE cells, the levels of Muc5ac protein remain unchanged as a consequence of increases in both translation and protein stability. Interestingly, some of the effects of DEX were opposite in a cell line.
A549 cells were cultured in 5% FBS for 12 h before treatment and in treatment. In ALI culture, HC was removed at day 0, and the cells were fed only from the bottom chamber. Throughout the remaining text, RTSE cell cultures represent submersed cultures of primary RTSE cells unless otherwise specified. A human adenocarcinoma cell line A549 was purchased from American Type Culture Collection (no. CCL-185; Manassas, VA) and cultured with DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**DEX treatment.** Before DEX treatment, submersed cultures of primary RTSE cells were depleted of endogenous GCs by removing HC from the medium on day 3 and stepwise reduction of FBS to 3% (day 3), 1% (day 5), and 0% (day 7). During serum depletion, the albumin concentration was maintained at the equivalent amount present in 5% FBS by addition of BSA. Cultures were maintained in serum-free and BPE-free complete medium for 24 h before DEX treatment. In ALI culture, HC was removed at day 0 and BPE removed 3 days before DEX treatment (day 5–7 of culture). Confluent A549 cells were cultured in 5% FBS for 12 h before treatment and in 2% FBS during DEX treatment. Cells were washed twice (submerged culture) or five times (ALI culture) with PBS before being exposed to various concentrations of DEX (10⁻⁹–10⁻⁶ M).

**Measurement of mucin synthesis and secretion by ELISA.** After DEX treatment, spent medium (SM) or apical washings (200 µl of PBS/well, 5 times) were collected, and the cells were lysed with 0.2 ml/well of PBS, pH 7.2, 1% Triton X-100, 2 mM EDTA, and 1 mM PMSE at 4°C for 10 min. SM, apical washings, and cell lysates were sonicated at 2,000 g for 5 min at 4°C, and the supernatants were stored at 80°C until assay. Protein concentrations were measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA) based on the method of Lowry et al. (39) using BSA as standard. High-molecular-weight (HMW) mucin content and specific MUC5AC/Muc5ac were quantified by ELISA as previously described using RT03 (50), a mouse monoclonal antibody that recognizes carbohydrate epitope(s) of HMW mucins produced by primary RTSE cells, and 45M1 (New Markers, Fremont, CA), a mouse monoclonal antibody reactive with the peptide core of human MUC5AC and rat Muc5ac. Briefly, SM, apical washings, and cell lysates were serially diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.5), and 100 µl/well were added to 96-well immunoplates (MaxiSorp surface; Nalge Nunc International, Rochester, NY) and incubated at 4°C overnight. Wells were blocked with 1% BSA and sequentially incubated at room temperature for 2 h with RT03 (50 µl/well, 1:10 dilution) or 45M1 (50 µl/well, 1:200), peroxidase-labeled goat anti-mouse antibody (100 µl/well, 1:3,000; KPL, Gaithersburg, MD), 3.3',5.5'-tetramethylbenzidine substrate (50 µl/well, KPL), and 1 N HCl stop solution. Absorbance was read at 450 nm with differential wavelength at 630 nm. The relative amount of mucins (HMW mucins or MUC5AC/Muc5ac) in each sample was determined from their ΔA₅₀₀ values using the linear portion of the standardized titration curves constructed with serial dilutions of reference SM, apical washing, or cell lysate samples. Reference titration curves were run simultaneously with the unknown samples during each experiment. In submersed RTSE cell cultures, to account for variation in cell numbers in individual wells, mucin index was calculated as previously described (28). Briefly, Muc5ac or total HMW mucin values from the SM or cell lysates of the 24-h DEX or vehicle-alone treatment samples were divided by the mucin values obtained from the SM of the 24-h pretreatment samples from the same culture well at the same dilution. By this method, each well served as its own control. Two negative controls were used in all experiments: 1) supplemented medium never exposed to A549 or RTSE cells and 2) an aliquot of test agents not exposed to the cells for possible ELISA interference.

**[^3H]Glucosamine labeling of HMW mucins and analysis of degradation fragments.** To assess the potential of DEX to affect degradation of HMW mucins produced by RTSE cells, mucins were metabolically radiolabeled for 24 h by incubating confluent submersed cultures in 24-well plates with 0.2 ml/well of serum-free complete medium containing 10 µCi/ml of D-[6-^3H]Glucosamine (60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) as previously described (26). SM was collected, digested with Streptomyces hyalurolyticus hyaluronidase (100 U/ml) at 37°C for 16 h, and applied to a Sepharose CL-4B (Pharmacia, Piscataway, NJ) column (1.0 x 45 cm) in 0.05 M sodium acetate, pH 7.0, containing 0.01% SDS (28). HMW mucins eluting in the void volume were collected, dialyzed against PBS, and quantified by liquid scintillation counting. To individual wells of confluent cultures of RTSE cells in 24-well plates, we added 1,000 U/ml of[^3H]glucosamine-labeled HMW (3H-HMW) mucins and incubated them for 0 or 24 h in the presence of vehicle control or 10⁻⁶ M DEX. [^3H]Glucosamine containing SM was collected and passed through a centrifugal filter device (Millipore) containing a 100,000-molecular-weight cut-off membrane at 3,000 g for 30 min at 4°C. Radioactivity in the upper and lower chambers was determined by liquid scintillation counting. The presence of degradation was also confirmed by Sepharose CL-4B column chromatography of the [^3H]-containing SM samples.

**Quantitation of MUC5AC and Muc5ac mRNAs by real-time PCR.** Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Ambion, Austin, TX) for 20 min at 37°C, and 1.0 µg was reverse transcribed at 42°C for 50 min in a 20-µl reaction mixture containing 0.5 mM dNTPs, 2.5 mM MgCl₂, 5.0 mM dithiothreitol, 50 ng random hexamer, and 200 U/µl of Superscript II reverse transcriptase (Invitrogen). The reaction was heat inactivated at 70°C for 15 min and digested with 1.0 µl of RNase H (1.0 U/µl) at 37°C for 20 min. Rat Muc5ac specific primers were designed using the sequence in GenBank (accession no. AB042530); sense strand, 5'-TAC CCC GAG CGT AGT GTA CC-3'; antisense strand, 5'-CAG GGG TCT TCA CAG AGC AT-3'; human MUC5AC specific primers were also designed using the sequence in GenBank (accession no. AA014101); sense strand, 5'-GCT TGC TCC GCC TAG ATG T-3'; antisense strand, 5'-AAC AGG CCC AGC CCT TAG AA-3'. GAPDH primers recognizing human and rat GAPDH mRNAs were designed according to the sequence in GenBank (accession no. J04308); sense primer, 5'-CAC TGGA GCA CAT TCC TTA C-3'; antisense primer, 5'-GGT ATG CAT GAT ACC CTT GCC-3'. PCR amplification was performed using a commercial kit (QuantiTect; Qiagen, Valencia, CA) in a LightCycler (Roche, Indianapolis, IN) using the following conditions: 95°C for 15 min and 40 cycles at 94°C for 15 s, 62°C (Muc5ac) or 60°C (MUC5AC and GAPDH) for 20 s and 72°C for 20 s. PCR detection threshold cycle (CT) values were calculated by LightCycler software, and differences between samples were calculated using the 2⁻ΔΔCT method (36).

**Pulse labeling and immunoprecipitation analysis of Muc5ac protein.** RTSE ALI cultures depleted of GCs and BPE were pretreated with 10⁻⁶ M DEX for 4 h, washed three times with 1-methionine-free DMEM, and incubated with [³⁵S]L-methionine for 2 h in 1-methionine-free DMEM supplemented with 4 mM L-glutamine plus all the additives for ALI complete medium except HC and BPE. At the end of the incubation, the apical surface was washed five times with 200 µl/well of PBS, and the cultures were pulsed with 80 µCi/well of [³⁵S]L-methionine (1,000 Ci/mmol; Moravek Biochemicals, Brea, CA) for 1 h in 0.5 ml/well of 1-methionine-free DMEM supplemented above the presence of either 10⁻⁶ M DEX or vehicle (ethanol). Cells were lysed on ice for 10 min with 100 µl/well of lysis buffer (40 mM phosphate buffer, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1% BSA, 2 mM EDTA, 0.5% NP-40, 0.5% sodium dodecyl sulfate). For immunoprecipitation, cell lysates were precleared with 5 µl of Protein A-Sepharose 4B (Pharmacia) for 1 h, washed three times with 200 µl of 1-methionine-free DMEM supplemented above, and separated by 7.5% SDS-PAGE. Membranes were incubated with 10 µg/ml of the rat Muc5ac polyclonal antibody (28). After washing, membranes were incubated with 10 µg/ml of [³⁵S]L-methionine (1,000 Ci/mmol; Moravek Biochemicals, Brea, CA) for 1 h in 0.5 ml/well of 1-methionine-free DMEM supplemented above the presence of either 10⁻⁶ M DEX or vehicle (ethanol). Centrifugation fragments.
0.25% deoxycholic acid, 1.0 mM PMSF, and 1.0% proteinase inhibitor cocktail). Lysates from three separate wells were pooled and centrifuged at 5,000 g for 5 min at 4°C. Equal protein quantities (20 μg) were diluted to 300 μl in lysis buffer containing 0.1% BSA and immunoprecipitated with 2 μg of 45M1 antibody and 40 μl of goat anti-mouse IgG1-agarose at 4°C for 16 h with continuous rotation, followed by centrifugation at 1,000 g for 2 min and washing five times with PBS, pH 7.2, and 0.05% Tween 20. Precipitates were resuspended in 30 μl of electrophoresis sample buffer (100 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 4.0% SDS, 20% glycerol, and 0.1% bromphenol blue), boiled for 3 min, and resolved on a 1.0% agarose gel (12 × 15 cm) in 40 mM Tris-acetate and 1 mM EDTA, pH 8.0, containing 0.1% SDS at 30 V for 16 h as previously described (53).

Proteins were fixed in 25% isopropanol and 10% glacial acetic acid for 45 min, and the gel was soaked for 30 min in Amplify fluorographic reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and dried under vacuum. Protein bands were visualized and quantified using a Fuji film FLA-5000 phosphoimager (Fuji, Stamford, CT).

Results

Results

**RESULTS**

**DEX suppresses Muc5ac mRNA expression but not Muc5ac protein levels in primary RTSE cells.** We determined the effect of DEX on Muc5ac mRNA levels and Muc5ac protein synthesis and secretion in submerged RTSE cells. As shown in Fig. 1, DEX significantly inhibited Muc5ac transcripts in RTSE cells compared with cells treated with vehicle alone but had no effect on GAPDH mRNA levels. Surprisingly, however, 24-h treatment of RTSE cells with DEX did not alter the levels of Muc5ac protein (Fig. 2A) or HMW mucins (Fig. 2B) in both cell lysates and SM. Identical results were observed when the treatment time was extended to 48 and 72 h (data not shown). The ratios of SM to cellular mucin levels were ~0.2 for the Muc5ac proteins (Fig. 2A) and 1.1 for HMW mucins (Fig. 2B), respectively. On the basis of our knowledge of the structure of airway mucins, this drastic difference in the ratio seems to be mainly due to the antibodies used; whereas Muc5ac mucins were quantified by 45M1 antibody that recognizes the peptide core of Muc5ac mucins, HMW mucins were quantified by RT03 antibody that recognizes sugar moieties of HMW mucins, including Muc5ac mucins, which are likely the major type of HMW mucins produced by RTSE cells. The results indicate that secreted mucins are much fewer in the number of molecules but fully glycosylated compared with cellular mucins that are at various stages of de novo synthesis, including glycosylation. DEX had no effect on total RTSE cell protein levels or cell viability during the culture period (data not shown).

To validate this finding, we repeated the experiment with primary RTSE cells grown at an ALI, a culture system resembling in vivo airway epithelium (21). Beginning at day 5, the apical washings of the ALI culture became highly viscous and contained large amounts of Muc5ac and HMW mucins (data not shown). Treatment with DEX for 24 h did not affect either constitutively secreted or cellular Muc5ac (Fig. 3A) or HMW mucins (Fig. 3B) but significantly reduced Muc5ac mRNA levels when tested at 10^{-7} M and 10^{-6} M (Fig. 3C).

We considered the possibility that lack of an inhibitory effect by DEX on Muc5ac and HMW mucin levels was masked by enzymatic degradation (e.g., cellular glycosidases and/or proteases) before ELISA analysis. To assess mucin degradation in RTSE cell culture SM, 3H-HMW mucins purified by gel filtration chromatography were added to confluent RTSE cell cultures, incubated for 0 or 24 h in the presence or absence of DEX, collected, and passed through a 100,000-molecular-weight cut-off membrane to separate intact mucins from degradation fragments. As shown in Table 1, this experiment revealed that there were no differences in the ability of the 3H-HMW mucins to pass through the membrane after 24 h of incubation in the presence or absence of DEX for 0 or 24 h. Sepharose CL-4B column chromatography of the 3H-containing SM resulted in a complete recovery of the 3H counts in the void volume fractions, confirming the above notion that secreted HMW mucins are not degraded during the 24-h culture period by RTSE cells regardless of the DEX treatment.

We also examined whether DEX affected mucin secretion stimulated through the P_{2A} receptor pathway. We (27) and others (10) previously showed that mucin release from airway goblet cells was stimulated by UTP. RTSE cells were cultured in the presence or absence of DEX for 24 h, the cells were washed and incubated for 30 min in the presence or absence of 0.2 mM UTP, and both Muc5ac and HMW mucins were quantified in the SM. As shown in Fig. 4, UTP stimulated release of both Muc5ac and HMW mucins by 71 ± 12% and 57 ± 6% for the control group and 65 ± 9% and 65 ± 10% for the DEX-treated group. Thus DEX treatment did not significantly (P > 0.05) affect the UTP-induced secretion of these...
mucins. Combined with the data presented above, these results indicated that DEX treatment affected neither constitutive nor stimulated mucin secretion by cultured RTSE cells.

**DEX increases translation and stability of Muc5ac protein in RTSE cells.** Two possible mechanisms may have accounted for our observations that DEX inhibited rat Muc5ac mRNA levels without altering Muc5ac protein levels: increased Muc5ac translation and/or increased Muc5ac protein stability. To examine the effect of DEX on Muc5ac translation, RTSE cells were subjected to pulse analysis with [35S]methionine in the presence or absence of 10\(^{-6}\) M DEX and [35S]methionine-labeled Muc5ac resolved on an agarose gel and quantified. As shown in Fig. 5A, under reducing conditions, agarose gel electrophoresis of the 45M1 antibody immunoprecipitates revealed two bands, the lower, fast migrating band probably representing nonglycosylated Muc5ac protein and the upper dispersed band representing fully glycosylated and partly glycosylated Muc5ac as previously described (49, 57). Quantification of the intensity of the two bands revealed a 2.6-fold increased [35S]methionine-labeled Muc5ac in the DEX-treated cells compared with vehicle control (Fig. 5B), indicating that DEX treatment enhanced the translation of Muc5ac protein.

To examine the effect of DEX on Muc5ac protein stability, RTSE cells were cultured in the presence or absence of 10\(^{-6}\) M DEX for 24 h. The cells were washed and treated for various
Confluent rat tracheal surface epithelial (RTSE) cells were metabolically labeled with \[^{3}H\]glucosamine for 24 h, spent medium (SM) was collected, and \[^{3}H\] glucosamine-labeled high-molecular-weight (\[^{3}H\]-HMW) mucins were purified from SM as described in MATERIALS AND METHODS. Aliquots of 1,000 dpm of \[^{3}H\]-HMW mucins were incubated in confluent RTSE cell cultures for 0 or 24 h in the presence of vehicle control or 10\(^{-6}\) M dexamethasone (DEX), SM was collected and subjected to centrifugation using a filter (100,000 molecular weight cut-off), and the radioactivity in each chamber was measured as described in MATERIALS AND METHODS. Values represent the means ± SE of 3 experiments. *Significantly increased Muc5ac glycoprotein in the DEX treatment group compared with the control group at the same time point (P < 0.05).

### Table 1. DEX does not affect degradation of secreted HMW mucins in primary RTSE cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Top chamber</th>
<th>Bottom chamber</th>
<th>Top chamber</th>
<th>Bottom chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>944.0±65.2</td>
<td>0.0</td>
<td>896.9±38.3</td>
<td>37.1±3.3</td>
</tr>
<tr>
<td>DEX</td>
<td>973.4±49.1</td>
<td>0.0</td>
<td>932.3±56.7</td>
<td>29.6±1.7</td>
</tr>
</tbody>
</table>

Confluent rat tracheal surface epithelial (RTSE) cells were metabolically labeled with \[^{3}H\]glucosamine for 24 h, spent medium (SM) was collected, and \[^{3}H\] glucosamine-labeled high-molecular-weight (\[^{3}H\]-HMW) mucins were purified from SM as described in MATERIALS AND METHODS. The amounts of 3H-HMW mucins in the top chambers of any of the 4 treatment groups were quantified by ELISA as described in MATERIALS AND METHODS. Each point represents means ± SE of 4 samples. *Significantly increased Muc5ac protein in the cultures were measured by ELISA using 45M1 antibody as described in MATERIALS AND METHODS. A: Muc5ac proteins were immunoprecipitated with 45M1, resolved on a 1% agarose gel, and visualized by a phosphoimager. Arrows indicate the glycosylated (top) and nonglycosylated (bottom) forms of \[^{35}S\]methionine-labeled Muc5ac protein. B: intensity of the bands in A were quantified by phosphoimage analysis and expressed as the mean values ± SE of 3 experiments. *P < 0.05 compared with vehicle control.

![Fig. 4](http://ajplung.physiology.org/)

**Fig. 4.** DEX does not affect UTP-stimulated secretion of Muc5ac and HMW mucins by RTSE cells. Confluent submerged cultures of RTSE cells were treated for 24 h with vehicle control or 10\(^{-6}\) M DEX. The cells were washed and incubated for 30 min in the absence (−) or presence (+) of 0.2 mM UTP. A and B: Muc5ac and HMW mucins in the SM were measured by ELISA as described in Fig. 2. Each bar represents means ± SE of 4 samples. *Significantly increased Muc5ac or HMW mucin secretion induced by UTP compared with the vehicle control (P < 0.05). There were no significant differences in UTP-induced Muc5ac or HMW mucin secretion between the control and DEX-treated groups (P > 0.05).

![Fig. 5](http://ajplung.physiology.org/)

**Fig. 5.** DEX enhances Muc5ac translation in RTSE cells. Hydrocortisone- and bovine pituitary extract-deprived RTSE cells (day 7) grown at ALI were labeled for 1 h with \[^{35}S\]methionine in the presence or absence of 10\(^{-6}\) M DEX as described in MATERIALS AND METHODS. A: Muc5ac proteins were immunoprecipitated with 45M1, resolved on a 1% agarose gel, and visualized by a phosphoimager. Arrows indicate the glycosylated (top) and nonglycosylated (bottom) forms of \[^{35}S\]methionine-labeled Muc5ac protein. B: intensity of the bands in A were quantified by phosphoimage analysis and expressed as the mean values ± SE of 3 experiments. *P < 0.05 compared with vehicle control.

![Fig. 6](http://ajplung.physiology.org/)

**Fig. 6.** DEX increases the stability of Muc5ac protein in primary RTSE cells. Because a majority of cellular proteins are degraded by the proteasomal pathway, one possible mechanism to account for the increased Muc5ac protein stability could be inhibition of the proteasome pathway by DEX. To test this possibility, GC-deprived RTSE cells were treated with CHX in the presence or absence of the proteasome inhibitors MG-132 or LLnL, and cellular levels of Muc5ac were monitored at various time lengths of time with 10 μg/ml of CHX to block de novo protein synthesis, and intracellular Muc5ac levels were quantified by ELISA. Our preliminary results confirmed that this concentration of CHX completely inhibited \[^{14}C\]threonine incorporation into HMW mucins within 1 h (data not shown). As shown in Fig. 6, in the absence of DEX treatment, the levels of intracellular Muc5ac decreased linearly over time in the presence of CHX such that by 24 h, the mucin level had decreased to ~41% of its pretreatment level. However, DEX treatment significantly delayed the CHX-mediated decline in cellular Muc5ac levels compared with vehicle alone-treated cells, achieving only ~20% reduction by 24 h. Based on the time of CHX treatment necessary for Muc5ac levels to reach 50% of their original values, we estimated the half-life of the Muc5ac pool to be 38 h in the presence of DEX and 20 h in the absence of DEX.
As can be seen in Fig. 7A, neither inhibitor affected the rate of CHX-induced decline in Muc5ac protein levels. These results indicated that cellular Muc5ac was not degraded by the proteasome pathway. To verify the effectiveness of the proteasome inhibitors, we measured IL-8 release by MG-132 as a positive control based on a recent report (60). Figure 7B shows that MG-132 increased IL-8 release by A549 cells in a dose-dependent manner, whereas neither the cellular nor secreted MUC5AC levels were affected by MG-132. Although we cannot rule out the possibility of differential sensitivity of RTSE and A549 cells to MG-132, these results indicate that the increase in Muc5ac protein stability by DEX was not likely due to the inhibition of the proteasomal degradation pathway.

Together, these results suggest that the inability of DEX to inhibit Muc5ac protein levels in RTSE cells, in spite of the significant suppression of its mRNA, was due to increased Muc5ac translation and intracellular protein stability.

DEX suppresses MUC5AC mRNA expression and protein secretion in A549 cells. Previous studies with NCI-H292 cells, a lung adenocarcinoma cell line, demonstrated that DEX suppressed mucus production as well as MUC2 and MUC5AC gene expression (22). To determine whether a similar effect occurred in another human airway cancer cell line, we treated A549 cells with 10⁻⁹ to 10⁻⁶ M DEX for 24 h and measured MUC5AC mRNA by real-time PCR and MUC5AC transcripts in a dose-dependent manner compared with cells treated with vehicle (ethanol) alone. The decrease in mRNA levels was statistically significant at all DEX concentrations tested (P < 0.05). DEX had no effect on GAPDH mRNA levels. Similarly, DEX significantly inhibited MUC5AC protein secretion but increased cellular MUC5AC levels compared with cells treated with vehicle alone (Fig. 8B). The amount of MUC5AC secreted during the 24-h period was ~6% of the total cellular MUC5AC. Total (cellular + secreted) MUC5AC protein levels were slightly greater in DEX-treated cells than those in control-treated cells (Fig. 8B), indicating that the synthesis of MUC5AC protein was slightly increased in the presence of DEX in A549 cells. However, total cellular protein contents were not significantly affected by DEX (Fig. 8B), indicating that its effect on MUC5AC is likely specific in this cell line. There was neither increased cell floating nor reduced cell viability, the latter being assessed by measurement of the percent of lactic dehydrogenase activity in the SM compared with the total activity in the culture (date not shown).

DISCUSSION

The major findings of our studies are that DEX treatment of primary RTSE cells had the following effects: 1) inhibition of Muc5ac mRNA levels; 2) increased translation of Muc5ac points. As can be seen in Fig. 7A, neither inhibitor affected the rate of CHX-induced decline in Muc5ac protein levels. These results indicated that cellular Muc5ac was not degraded by the proteasome pathway. To verify the effectiveness of the proteasome inhibitors, we measured IL-8 release by MG-132 as a positive control based on a recent report (60). Figure 7B shows that MG-132 increased IL-8 release by A549 cells in a dose-dependent manner, whereas neither the cellular nor secreted MUC5AC levels were affected by MG-132. Although we cannot rule out the possibility of differential sensitivity of RTSE and A549 cells to MG-132, these results indicate that the increase in Muc5ac protein stability by DEX was not likely due to the inhibition of the proteasomal degradation pathway.

Together, these results suggest that the inability of DEX to inhibit Muc5ac protein levels in RTSE cells, in spite of the significant suppression of its mRNA, was due to increased Muc5ac translation and intracellular protein stability.
protein; and 3) increased stability of intracellular Muc5ac protein. The net effect of decreased Muc5ac mRNA levels and increased protein translation/stability was to maintain relatively constant levels of intracellular Muc5ac content. Similar results were observed with total HMW mucins. In contrast, DEX suppressed MUC5AC mRNA levels and MUC5AC protein secretion by A549 human adenocarcinoma cells. DEX did not affect constitutive or UTP-stimulated mucin secretion by RTSE cells. Although MUC5B has been identified to also be an important marker of goblet cell hyperplasia and mucus hypersecretion (4, 9, 48, 54), our initial focus on MUC5AC/ Muc5ac was prompted by the previous report describing down-regulation of this mucin following DEX treatment of NCI-H292 cells (22) and the unavailability of antibodies specific for rat Muc5b.

To date, GCs are the only class of drugs available to clinically control mucus hypersecretion associated with airway inflammation. Because proinflammatory cytokines are well known to stimulate mucin gene transcription and mucus production (3, 8, 15, 29, 37, 38, 51, 59), it is not unreasonable to speculate that the mechanism of the inhibitory effects of GCs on mucus hypersecretion by airway epithelial cells involves an indirect effect of their anti-inflammatory properties (25). Reports by others, however, also suggested a direct inhibitory action by GCs on mucus-secreting cells, although these studies are controversial. Kai et al. (22) reported that DEX suppressed MUC5AC mRNA as well as MUC5AC protein levels by NCI-H292 cells. Additionally, GCs were shown to inhibit mucin glycoprotein synthesis by guinea pig gall bladder explants (40) and isolated human gastric mucosa (43) and reduce Muc5ac mRNA levels in rat gastric mucosal cell cultures (52). Conversely, Gollub et al. (16) showed increased MUC5AC transcripts induced by DEX in a human uterine epithelial cell line, and Finnie et al. (13) observed elevated mucin biosynthesis stimulated by corticosteroids in colonic epithelial biopsies. Finally, DEX treatment of human primary nasal epithelial cells was reported to have no effect on MUC5AC or MUC5B mRNA levels (19).

The MUC5AC gene promoter contains a GC responsive element as well as multiple binding sites for NF-κB, the latter previously shown to be negatively regulated by GCs (34, 42). Thus it was not unexpected that DEX downregulated MUC5AC gene transcription by A549 cells. However, we were intrigued to note that whereas DEX suppressed rat Muc5ac transcripts in primary RTSE cells, it had no effect on either the secreted or the cellular levels of Muc5ac or HMW mucin proteins, unlike the previous report on a human lung epithelial cell line (22). Two possible mechanisms will likely explain our finding: 1) an increase in translation and/or 2) a positive effect on protein stability.

Our pulse study clearly demonstrated that DEX increased newly synthesized Muc5ac levels. It has been shown that the 5′-untranslated (5′-UT) region is important for the formation of mRNA initiation complex, which modulates translation efficiency. Putative steroid-modulatory elements have been identified in the 5′-UT region of mRNAs for myelin basic protein (56) as well as Na-K-ATPase α1- and α2-subunits (11). Whether a similar module exists in the 5′-UT region of Muc5ac and, if so, whether its translation is affected by DEX through this mechanism, remain to be explored.

Our results also demonstrated that DEX increases the cellular Muc5ac protein stability in primary RTSE cells. Recently, it has been shown that neutrophil elastase increases the mRNA stability of MUC5AC (57) as well as MUC4 (14). Although a great number of studies focused on the transcriptional and mRNA stability regulation of the mucin proteins, no information is available on the regulation of mucin protein stability. It has been reported that DEX reduces the chymotrypsin-like activity associated with 26S proteasomes in thymocytes (2), whereas it protects MAP kinase phosphatase-1 from proteasome-mediated degradation (23). Therefore, we thought the increase in Muc5ac protein stability by DEX could be due to an inhibition of proteolysis through the proteasome pathway, which is known to be responsible for the degradation of 80–90% of cellular proteins (32). We used two proteasome inhibitors that are widely used for cultured cells (32). Both are highly potent, reversible, and relatively nontoxic (31, 32, 46). Our results indicate that normally, Muc5ac protein is not degraded by the proteasome, and the increase in the stability of Muc5ac by DEX does not involve the proteasomal degradation pathway. Alternatively, the GCs may activate expression of antiproteases (1, 30), thereby blocking Muc5ac intracellular proteolysis and increasing its half-life. Finally, the lysosomal proteolytic pathway might be another possible mechanism for the altered Muc5ac stability induced by DEX.

In either case, it is apparent that the net effect of DEX on mucin production/secretion involves the combination of reduced gene transcription and increased translation and protein stability. In the case of primary RTSE cells, these opposing effects are relatively similar in magnitude such that the combined effect is to maintain constant Muc5ac levels. Current studies in our laboratory are directed at further elucidating the molecular mechanisms of mucin transcription, translation, and secretion following DEX treatment.

Finally, it is worth noting that immortalized cell lines could behave quite differently from primary cells since they have been adapted to nonphysiological environments under constant pressure. The results from the present study clearly support this notion. Whereas secretion of MUC5AC or HMW mucins from the widely used lung epithelial cell lines was inhibited by DEX in a dose-dependent manner, that which was from the primary airway epithelial cell cultures, both submerged and ALI cultures, was not affected by DEX. Such a dramatic contrast in the pharmacological responses among the different cell types seems to indicate that extreme caution is needed when interpreting results obtained from pharmacological studies of cell lines.

ACKNOWLEDGMENTS

We thank Dr. James Lee for providing technical assistance in real-time PCR.

GRANTS

This work was supported, in part, by grants from the Cystic Fibrosis Foundation and the Maryland Industrial Partnerships.

REFERENCES


