Chronic Intermittent Mechanical Stress Increases MUC5AC Protein Expression

Jin-Ah Park¹ and Daniel J. Tschumperlin¹

¹Molecular and Integrative Physiological Sciences Program, Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts

Increased abundance of mucin secretory cells is a characteristic feature of the epithelium in asthma and other chronic airway diseases. We showed previously that the mechanical stresses of airway constriction, both in the intact mouse lung and a cell culture model, activate the epidermal growth factor receptor (EGFR), a known modulator of mucin expression in airway epithelial cells. Here we tested whether chronic, intermittent, short-duration compressive stress (30 cm H₂O) is sufficient to increase the abundance of MUC5AC-positive cells and intracellular mucin levels in human bronchial epithelial cells cultured at an air-liquid interface. Compressive stress applied for 1 hour per day for 14 days significantly increased the percentage of cells staining positively for MUC5AC protein ($22.0 \pm 3.8\%$, mean $\pm$ SD) relative to unstimulated controls ($8.6 \pm 2.6\%$), and similarly changed intracellular MUC5AC protein levels measured by Western and slot blotting. The effect of compressive stress was gradual, with significant changes in MUC5AC-positive cell numbers evident by Day 7, but required as little as 10 minutes of compressive stress daily. Daily treatment of cells with an EGFR kinase inhibitor (AG1478, 1 μ M) significantly but incompletely attenuated the response to compressive stress. Complete attenuation could be accomplished by simultaneous treatment with the combination of AG1478 and a transforming growth factor (TGF)- β_2 (1 μ g/ml)-neutralizing antibody, or with anti-TGF- β_2 alone. Our findings demonstrate that short duration episodes of mechanical stress, representative of those occurring during bronchoconstriction, are sufficient to increase goblet cell number and MUC5AC protein expression in bronchial epithelial cells *in vitro*. We propose that the mechanical environment present in asthma may fundamentally bias the composition of airway epithelial lining in favor of mucin secretory cells.

Keywords: asthma; mechanotransduction; EGFR; TGF- β ; bronchial epithelium

The mucus lining of the airways provides an important front line defense against the inhaled environment (1, 2). Secreted high-molecular-weight, highly glycosylated mucin proteins, primarily products of *MUC5AC* and *MUC5B* genes, create a hydrated polymer gel on the airway surface to which inhaled particles stick, enabling clearance via the mucociliary escalator (1). However, mucin hypersecretion, driven in part by metaplasia and hyperplasia of mucin secretory cells, is a common occurrence in chronic airway diseases such as asthma and chronic obstructive pulmonary disease (3, 4). Excessive secretion of mucin and accompanying alterations to mucus mechanical properties can cause airway obstruction and serve as a niche

for bacterial growth, contributing to the morbidity and mortality of airway diseases (1–8). Hence, there is a strong interest in understanding common and disease-specific mechanisms leading to goblet cell hyperplasia and mucin hypersecretion (1, 2, 9–12).

Asthma is characterized physiologically by episodic reversible airway obstruction, attributable in part to muscular constriction of the airways. We have shown in both intact mouse airways, and in human cell culture, that the compressive mechanical stresses that accompany airway constriction can trigger activation of the epidermal growth factor receptor (EGFR) in airway epithelium (13). The EGFR is known to be a key mediator of mucin gene expression and goblet cell differentiation (9, 14–21). Thus we hypothesized that episodic compression of airway epithelial cells would lead to increased abundance of goblet cells through EGFR signaling. We demonstrate here that daily exposure to physiologic levels of compressive stress for as little as 10 minutes to 1 hour is sufficient to significantly increase the fraction of MUC5AC-positive cells and levels of intracellular MUC5AC protein in well differentiated primary human airway epithelial cultures. This is the first demonstration that alterations of the mechanical environment may influence the cellular composition of the airway epithelium. Our findings support a role for the mechanical environment present in asthma, characterized by intermittent episodes of compressive stress, in shifting epithelial cells toward a mucin secretory phenotype, potentially amplifying and prolonging the effects of acute airway narrowing.

MATERIALS AND METHODS

Cell Culture

Normal human bronchial epithelial cells (Clonetics, Lonza, Walkersville, MD) were expanded on tissue culture treated plastic in bronchial epithelial basal media (BEBM) supplemented with bovine pituitary extract (BPE, 52 μ g/ml), hydrocortisone (0.5 μ g/ml), human epidermal growth factor (hEGF, 0.5 ng/ml), epinephrine (0.5 μ g/ml), insulin (5 μ g/ml), triiodothyronine (6.5 ng/ml), transferrin (10 μ g/ml), gentamicin (50 μ g/ml), amphotericin-B (50 ng/ml), bovine serum albumin (1.5 μ g/ml), and retinoic acid (50 nM). Cells at passage 2 or 3 were then transferred to microporous polyester inserts (0.4 μ m pore size, Transwell-Clear; Corning Costar, Corning, NY) and fed with a 1:1 mixture of BEBM and Dulbecco's Modification of Eagle's Media (DMEM; Mediatech, Herndon, VA) supplemented with the same components detailed above and as previously described (22). Media was applied apically and basally until the cells were confluent and then basally after an air-liquid interface (ALI) was established. Cells were cultured at ALI for 14 days to promote relatively stable expression of goblet and ciliated cells before chronic mechanical stress exposure (22, 23).

Mechanical Stress

To expose cells to mechanical stress, silicon plugs with an access port for pressure application were press fit into the top of each Transwell, creating a sealed pressure chamber over the apical surface of the NHBE cells (24, 25). Each plug was connected to a 5% CO₂ (balance room air) pressure cylinder via a humidified chamber maintained at 37°C. The pressure in the apical chamber was increased by 30 cm H₂O

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Correspondence and requests for reprints should be addressed to Daniel J. Tschumperlin, Ph.D., Molecular and Integrative Physiological Sciences Program, Harvard School of Public Health, 665 Huntington Ave., SPH1-309, Boston, MA 02115. E-mail: dtschump@hsph.harvard.edu

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for the indicated duration, while the basal surface and medium remained at atmospheric pressure. The resulting apical-to-basal transcellular pressure produced a continuous compressive stress (30 cm H₂O) comparable to that present in the airway epithelium during bronchoconstriction, orders of magnitude higher than the stress experienced by the airway epithelium during breathing (24, 26). NHBE cells were subjected to variable durations (10 min or 1 h) of once daily transcellular pressure beginning at ALI Day 14, and continuing for up to 14 additional days; control cells were treated similarly including placement in the experimental apparatus, but were not exposed to the pressure gradient (Figure 1A).

Inhibitor Treatment

In inhibitor studies, cells were pre-treated each day with the epidermal growth factor receptor tyrosine kinase inhibitor AG1478 (1 μ M; Calbiochem, San Diego, CA) alone, a neutralizing antibody to transforming growth factor (TGF)- β_2 (1 μ g/ml; R&D Systems, Minneapolis, MN) alone, or both in combination beginning at ALI Day 14. Thirty minutes after addition of inhibitors, compressive stress was applied. IgG (1 μ g/ml; R&D Systems) was used as the negative control for the effect of TGF- β_2 antibody. Inhibitors were applied to cells at their final concentration in normal feeding media. Control wells with no inhibitor were fed at the same time before application of pressure, and inhibitor controls were treated with inhibitor on each day but not exposed to compressive stress. Experiments were repeated a minimum of two times, using three to six replicate wells in each independent experiment.

MUC5AC Immunofluorescence

NHBE cells were fixed in 3% paraformaldehyde for 20 minutes and then treated with 1% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes. Nonspecific immunoglobulin binding was blocked by incubation with 10% normal goat serum in PBS with 2% bovine serum albumin (BSA) for 1 hour at room temperature. A mouse monoclonal antibody against mucin 5AC (MUC5AC, 1:500 dilution, clone 45M1; Neo Markers, Fremont, CA) was diluted in PBS with 2% BSA and incubated at 4°C overnight. The samples were then incubated in a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG [H + L] conjugate; Molecular Probes Inc., Eugene, OR) diluted 1:700 in PBS at room temperature for 2 hours. For nuclear counterstaining cells were incubated in 1 μ g/ml of propidium iodide (Sigma, St. Louis, MO) in PBS for 5 minutes. Controls in the absence of primary antibodies confirmed the specificity of the immunolabeling (data not shown).

Confocal microscopy was performed to capture paired red (nuclear staining) and green (anti-MUC5AC) channel x-y images of immunostained NHBE cells; paired images were captured for each channel in the plane of maximal signal intensity. The microscopist was blinded to the sample conditions, and acquired six paired red and green channel images randomly from each well at low power for further analysis.

Image Analysis

To automate the images analysis procedure, a custom-written script was developed using IGOR Pro software (Wavemetrics, Lake Oswego, OR). Raw single plane confocal images were transformed into grayscale and subjected to thresholding to segment images (Figure 1B). The threshold intensity for positive staining was experimentally determined in a sample set of images by comparing manually counted cells to the algorithm output for various threshold values; this process was repeated for both propidium iodide and anti-MUC5AC staining. To maintain consistency, we retained the same channel-specific threshold values for all subsequent image analysis. The script analyzes each segmented image to identify contiguous areas of positive staining pixels. To account for noise and multiple positively staining cells merged together in segmented images, we instituted a minimum size criterion to exclude small objects (likely noise) and a maximal size criterion based on observations of typical cell size. Contiguous areas larger than this size were subdivided by the typical cell size to approximate the number of cells in such a cluster. We confirmed the validity of this approach by comparing manual cell counts and algorithm results in a set of sample images (Figure 1C).

Real-Time rt-PCR

At various time intervals (2, 4, 8, 18, and 24 h) after a single episode of compressive stress (1 h), or at 24 hours after the last of 14 days of chronic intermittent compressive stress, RNA was purified from cell lysates with RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Two micrograms of total RNA was used for synthesizing cDNA using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). Twenty nanograms of cDNA was used for the real-time PCR reaction in the mixture of primers and the 2 $\times$ SYBR green PCR master mix. The forward and reverse primers for the target genes and β -actin endogenous control were from published reports (21, 27), or were generated by Primer Express 3.0 software (Table 1). All primer sets used in this study were validated against β -actin using various amount of cDNA to ensure similar levels of PCR efficiency. Fold changes were calculated by the comparative delta-delta Ct method (28).

Figure 1. Experimental design and methods. (A) Normal human bronchial epithelial (NHBE) cells were differentiated to a mucociliary phenotype in defined media at an air-liquid interface for 14 days before mechanical stimulation. Compressive stress (30 cm H₂O) was applied for 1 hour or 10 minutes per day, for up to 14 consecutive days. Cells were fixed for immunohistochemistry (x) after 3, 7, or 14 days of chronic episodic compressive stress. RNA for qPCR was isolated from cells at various intervals after a single 1-hour compressive stress, or 24 hours after the final (14th) day of chronic compressive stress (o). (B) Fixed NHBE cells were double stained with propidium iodide (PI, nuclear counterstain) and anti-MUC5AC. Primary x-y single plane confocal images were subjected to automated segmentation by thresholding and counting with minimal and maximal size criteria to exclude noise and account for merged appearance of neighboring cells in segmented images. (C) Quantitative comparison of manual cell counting and automated counting algorithm in small sample set of PI and anti-MUC5AC images (mean $\pm$ SD, $n = 5$ images per condition).

TABLE 1. REAL-TIME PCR PRIMERS

Gene	Primers		Reference
<i>MUC5AC</i>	5'-GGAAGTGTGGGACAGCTCTT-3'	Forward	(27)
	5'-GTCACATTCTCAGCGAGGTC-3'	Reverse	
<i>FOXA2</i>	5'-TCTTAAGAAGACGACGGCTTCAG-3'	Forward	(21)
	5'-TTGCTCTCTCATTGCTCTCGAT-3'	Reverse	
<i>FOXJ1</i>	5'-ATCCGCCACAACCTGTCTCT-3'	Forward	Primer express 3.0
	5'-CTTGCCTGGTTCGCTCTCTC-3'	Reverse	
<i>Tektin-1</i>	5'-GCCCTTGACATCACTGAGA-3'	Forward	Primer express 3.0
	5'-TCAATGCCAATGCGCTTCT-3'	Reverse	
β -actin	5'-CTGGAACGGTGAAGGTGACA-3'	Forward	(27)
	5'-AAGGGACTTCTGTAAACAATGCA-3'	Reverse	

Western Blotting

Cells were lysed with 2× sample buffer (125 mM Tris-Cl, pH 6.8), 25% glycerol, 4% sodium dodecyl sulfate, 10% β -mercaptoethanol, 0.04% bromophenol blue), boiled for 10 minutes, loaded on precast 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels, and transferred to a polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell BioScience, Inc., Keene, NH). The PVDF membranes were incubated with 5% nonfat milk, and probed with the primary antibodies against β -tubulin IV (1:500, Sigma) or β -actin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) for the loading control. After incubation with secondary antibody conjugated with HRP, membranes were briefly incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotech, Rockford, IL) and protein bands were visualized by the ChemiGenius Bioimaging system (Syngene, Frederick, MD). The relative amount of β -tubulin IV was analyzed using GeneSnap software (Syngene) and normalized to the amount of β -actin.

For the semiquantitative Western blot analysis of the MUC5AC protein, the cells were lysed with 2× sample buffer omitting β -mercaptoethanol to avoid destruction of the epitope recognized by the MUC5AC monoclonal antibody (1:500, clone 45M1; Neo Markers). Protein lysates were loaded on 0.8% agarose gels in 1× TAE buffer (pH 8.5) containing 0.1% SDS, separated overnight, and transferred to PVDF membranes (29). The rest of the process for Western blot analysis was the same as described above.

Further quantification of intracellular MUC5AC protein was performed by slot blotting (30). To establish the linear range of the slot blot assay, serially diluted cell lysates were loaded on the PVDF membrane assembled in a Bio-Dot SF apparatus (Bio-Rad Laboratories, Hercules, CA) and vacuum applied for 1 minute followed by the same immunodetection protocol as Western blotting of MUC5AC. The linear range of the assay was found to correspond to dilutions of 4- to 32-fold. Subsequent quantification of MUC5AC protein levels in experimental and control conditions was performed using 16-fold dilutions to ensure accurate measurements of relative MUC5AC protein abundance.

Statistical Analysis

Data are presented as mean $\pm$ SD, and where indicated were analyzed by Student's *t* tests (*P* values < 0.05 considered significant), with Bonferroni correction applied for multiple comparisons.

RESULTS

Episodic Compressive Stress Enhances MUC5AC Protein Expression

NHBE cells were grown at air-liquid interface in defined medium for 14 days to establish a mature mucociliary phenotype featuring both goblet and ciliated cells (22, 23). Over the subsequent 14 days the proportion of control cultures staining positively for MUC5AC remained stable between 5 and 10% (Figures 2A and 2B). In contrast, paired cultures exposed to episodic compressive stress for 1 hour each day exhibited a statistically significant increase in the percentage of MUC5AC

positive cells after 7 days relative to time-matched controls ($11.7 \pm 2.8\%$ versus $5.6 \pm 1.1\%$), with a continued increase at 14 days ($22.0 \pm 3.8\%$ versus $8.6 \pm 2.6\%$). Notably, there was no difference between control and compression-exposed cultures after 3 days of episodic compression, implying that the MUC5AC response to mechanical stress was gradual and delayed in onset.

To further validate the increase in MUC5AC-positive cells in response to compressive stress, we compared responses to 14 days of episodic compressive stress (1 h/d) across cells grown from two different human donors. While the baseline percentage of MUC5AC-positive cells varied between these donors, both exhibited a statistically significant increase in response to episodic compressive stress with magnitude of the change greater than 2-fold in each donor (Figure 2C). After confirming the response to 1 hour/day compressive stress, we examined a shorter-duration stimulus (10 min/d), and found a similar increase in the percentage of MUC5AC-positive cells evoked by this shorter duration stimulus (Figure 2D). Strikingly, these results demonstrate that even 10 minutes of daily compressive stress, with cells maintained in a no-stress environment the remaining 23 hours and 50 minutes of the day, is sufficient to evoke an increase in MUC5AC-positive cells.

To confirm and extend our results, we also examined the effect of chronic mechanical stress on intracellular protein levels of MUC5AC, and for comparison measured levels of β -tubulin IV, a protein expressed in ciliated NHBE cells (22). Cell lysates were collected from wells that had been exposed to compressive stress for 1 hour/day for 14 days, and parallel unstressed control wells. Western blotting for MUC5AC qualitatively confirmed our immunostaining results, demonstrating a clear increase in intracellular MUC5AC protein levels in chronic mechanical stress samples relative to time-matched controls (Figures 3A and 3B). The results of Western blot analysis were confirmed quantitatively by slot blot analysis (Figures 3C and 3D). These intracellular protein results were from a third independent human donor distinct from those used in Figure 2, emphasizing the robustness of the MUC5AC response across multiple cell donors and outcome measures. In contrast to the prominent change in MUC5AC levels, we observed no statistically significant change in β -tubulin IV levels in mechanically stressed samples relative to time-matched controls.

Compressive Stress Effects on Transcript Levels of Epithelial Phenotypic Markers

To investigate whether changes in MUC5AC and other epithelial phenotypic markers were paralleled at a transcriptional level, RNA was collected 24 hours after the last of 14 consecutive days of chronic mechanical stress to measure cumulative effects of compressive stress on gene expression. Transcript levels of *MUC5AC*, *FOXA2*, *FOXJ1*, and *Tektin-1*

Figure 2. Chronic episodic compressive stress enhances MUC5AC expression. (A) Representative immunohistochemistry for MUC5AC from control wells and wells exposed to compressive stress for 1 hour/day, 14 days total. Scale bar is 20 μ m. (B) Percentage of MUC5AC+ cells present in NHBE cultures at 3, 7, and 14 days after initiation of episodic 1 hour/day compressive stress and time-matched controls ($*P < 0.05$ versus control). (C) Comparison of response to 14 days of episodic compressive stress (1 h/d) for two cell donors ($*P < 0.05$ versus control). (D) Comparison of response to 14 days of episodic compressive stress of duration 1 hour/day versus 10 minutes/day ($*P < 0.05$ versus control).

were examined by qPCR. *FOXA2*, a member of the forkhead box (FOX) family of transcription factors, is implicated in negative regulation of goblet cell metaplasia and hyperplasia, as the conditional knockout of *Foxa2* in mouse lung promotes increased goblet cell abundance (31). Supporting this connection, IL-13 stimulation of NHBE cells decreased *FOXA2* in an EGFR-dependent fashion in parallel with increased *MUC5AC* expression (21). *FOXJ1*, another member of the forkhead box family of transcription factors, is expressed in ciliated cells in airway epithelium (32) and plays a role in ciliogenesis during lung development (33). Deletion of *Foxj1* in mouse leads to the

failure of cilia formation (34). *Tektin-1* encodes a protein expressed in ciliary axoneme, and its expression in ciliated human bronchial epithelial cells has been demonstrated by proteomic analysis (32, 35). IL-13 treatment of cultured epithelial cells leads to decreased *FOXJ1* and *Tektin-1* transcript levels and loss of cilia in parallel with enhanced *MUC5AC* expression (36, 37). Thus, the results with IL-13 stimulation provide a basis for investigating whether MUC5AC responses to compressive stress are accompanied by changes in ciliary phenotypic markers. Surprisingly, in our chronic mechanical stress samples we were unable to detect significant changes in

Figure 3. Chronic episodic compressive stress alters MUC5AC but not β -tubulin IV expression. (A) Semiquantitative analysis of Western blotting for MUC5AC and β -tubulin IV from cells exposed to 14 days of episodic compressive stress, and time-matched controls (1 h/d) ($*P < 0.05$; data are from two independent experiments). (B) Representative Western blots of MUC5AC glycoprotein, β -tubulin IV, and β -actin loading control from two control wells, and four wells exposed to 1 hour/day episodic compressive stress for 14 days. (C) Determination of linear range of MUC5AC protein detection by slot blotting using serial dilutions (as indicated) of cell lysates. (D) Quantitative analysis of intracellular MUC5AC detected by slot blotting ($*P < 0.01$; data are from two independent experiments).

expression of any of these transcripts relative to time-matched controls (Figure 4A).

To gain a more detailed understanding of how transcripts for these phenotypic markers vary with compressive stress, we measured expression of the same panel of genes after a single one-hour exposure to compressive stress at 2, 4, 8, 18, and 24 hours after initiation of stress. Changes in transcript levels were gradual and greatest at 24 hours (data not shown), consistent with the kinetics of other mucin stimulatory responses (21); we therefore gathered additional data from two cell donors at 24 hours. While positive control stimulation with PMA at 24 hours enhanced *MUC5AC* transcript levels as expected (data not shown), there were no donor-specific statistically significant changes in transcript levels for *MUC5AC*, *FOXA2*, *FOXJ1*, and *Tektin-1* (Figure 4B). However, we did observe trends toward enhanced *MUC5AC* transcript levels and reduced *FOXA2* transcript levels that were consistent across both donors (Figure 4B). When replicate data from both donors were combined and analyzed together, the results reached statistical significance for *FOXA2* ($P = 0.017$, 0.80 ± 0.14 -fold change), and for *MUC5AC* the same analysis reached a P value of 0.08 (1.2 ± 0.23 -fold change).

Figure 4. Chronic and acute compressive stresses evoke modest changes in transcript levels for goblet and ciliated phenotypic markers. (A) Quantitative PCR analysis of *MUC5AC*, *FOXA2*, *FOXJ1*, and *Tektin-1* transcript levels from 14 days of chronic compressive stress samples (1 h/d, RNA isolated 24 h after final compressive stress exposure). For each gene transcript, levels are normalized to time matched control levels, both adjusted by endogenous control transcript expression. Data are from three independent experiments. (B) Quantitative PCR analysis of *MUC5AC*, *FOXA2*, *FOXJ1*, and *Tektin-1* transcript levels at 24 hours after a single 1-hour episode of compressive stress. For each donor and gene transcript, levels are normalized to time matched control levels, both adjusted by endogenous control transcript expression. Data are from three independent experiments per donor. When data from two donors are combined, *FOXA2* expression is significantly decreased by compressive stress (* $P = 0.017$), while P value for *MUC5AC* is 0.08.

EGFR and TGF- β_2 Contribute to the MUC5AC Response to Episodic Compressive Stress

We tested the role EGFR activation plays in transducing chronic intermittent compressive stress using the small molecule EGFR inhibitor AG1478. Daily pretreatment with AG1478 at a concentration that completely blocks mechanical stress induced EGFR activation (1 μ M [13], and data not shown) significantly attenuated the increase in MUC5AC-positive cells driven by compressive stress (1 h/d, 14 d; Figure 5A). AG1478 alone had minimal effect on baseline MUC5AC-positive cells relative to controls, indicating that the MUC5AC-expressing phenotype, once established in cultured airway epithelial cells, is relatively stable over 14 days, even in the presence of continuous EGFR blockade.

Given the incomplete attenuation of the MUC5AC response in the presence of EGFR blockade, we hypothesized that TGF- β_2 , a member of the TGF- β superfamily, could contribute to MUC5AC expression in compressed airway epithelial cells (38, 39). Daily administration of a neutralizing antibody to TGF- β_2 (1 μ g/ml) along with AG1478 completely attenuated the increased number of MUC5AC-positive cells in response to episodic compressive stress (1 h/d, 14 d) relative to inhibitor controls (Figure 5B). We further analyzed the effect of blocking TGF- β_2 alone with daily administration of neutralizing antibody, and observed complete attenuation of the changes in intracellular MUC5AC levels induced by chronic compressive stress; control treatment with IgG had no effect on the response to episodic compressive stress (Figure 5C).

DISCUSSION

The mechanical environment present in asthmatic airways is characterized by acute episodes of constriction that impose compressive stresses on the airway epithelium. These stresses can be of magnitudes up to 30 cm H₂O (24, 26), and of indeterminate duration, in contrast to the more modest magnitude stresses that accompany cyclic breathing (40). Our results demonstrate that compressive stress, when applied for as little as 10 to 60 minutes per day, is sufficient to enhance the abundance of MUC5AC-positive cells and intracellular levels of MUC5AC in primary bronchial epithelial cells. While MUC5AC expression and goblet cell hyperplasia have previously been attributed to growth factor and cytokine signaling downstream of various environmental and inflammatory stimuli (2, 8, 9, 12, 38, 41, 42), our results with episodic compression demonstrate comparable (~ 2 -fold) changes in MUC5AC expression, suggesting that the mechanical environment in the setting of airway constriction is a potentially potent modifier of epithelial composition. One unique aspect of our cell culture model is that we induced a relatively stable mucociliary phenotype (14 d ALI) before onset of experimental stimulation. Even in the context of an already differentiated epithelium, episodic mechanical stress was able to significantly alter the proportion of MUC5AC-positive cells. If corroborated in animal models and human subjects, our findings suggest that the airway narrowing caused by bronchoconstriction could be both amplified and prolonged by local mechanical stress-induced mucin synthesis and eventual hypersecretion (43).

The brief duration of daily mechanical stress needed to promote gradual increases in the MUC5AC-positive cell population was striking (Figure 2), but completely in keeping with our prior demonstration that the primary signaling response to sustained compressive stress is transient and largely complete within minutes (13, 25; D. J. Tschumperlin, unpublished observation). One explanation for the impressive response to such

Figure 5. MUC5AC response to compressive stress is driven by epidermal growth factor receptor (EGFR) and transforming growth factor (TGF)- β_2 . (A) Compressive stress elevated MUC5AC-positive cell fraction relative to time-matched control (* $P < 0.05$, control versus stress). EGFR small molecular inhibitor AG1478 (1 μ M) applied daily 30 minutes before episodic compressive stress (1 h/d, 14 d) significantly attenuated the MUC5AC response to compressive stress (* $P < 0.05$, stress versus AG1478 + stress). However, the response to compressive stress in the presence of AG1478 remained significant compared with AG1478 control (* $P < 0.05$, AG1478 control versus AG1478 + stress). AG1478 alone had insignificant effect on baseline percentage of MUC5AC-positive cells. (B) Combined treatment with AG1478 (1 μ M) and TGF- β_2 -neutralizing antibody (1 μ g/ml) completely attenuated MUC5AC response to episodic compressive stress (1 h/d, 14 d) (* $P < 0.05$, stress versus AG1478 + anti-TGF- β_2 + stress). The combined treatment alone did not significantly alter baseline percentage of MUC5AC-positive cells. (C) TGF- β_2 -neutralizing antibody alone significantly attenuated intracellular MUC5AC protein level response to compressive stress, while IgG showed no effect (* $P < 0.01$, control versus stress; # $P < 0.05$, anti-TGF- β_2 + stress versus stress, anti-TGF- β_2 + stress versus IgG + stress).

brief stimulation is that EGFR activation triggers enhanced expression of EGF family ligands (44), initiating a positive feedback loop promoting an amplified and prolonged secondary wave of EGFR activation. The sensitivity of the MUC5AC response to EGFR activation likely reflects the important innate defense function of the mucus lining layer (1), and the

common role the EGFR plays in transducing multiple diverse environmental stimuli into this innate defense response (9). Moreover, the gradual nature of the increase in MUC5AC-positive cells we observed is consistent with the complexity of the cellular processes involved in committing to a specialized mucin secretory state (23).

While blockade of EGFR signaling was able to significantly attenuate the MUC5AC response to chronic mechanical stress, consistent with its pivotal role in transducing other environmental stimuli (9, 17), the attenuation was incomplete (Figure 5A). We evaluated TGF- β_2 as an additional potential mediator of compressive stress induced MUC5AC expression based on our prior demonstration of TGF- β_2 secretion in response to compressive stress in NHBE cells (39), and on the work of Chu and coworkers showing that TGF- β_2 levels are elevated in asthmatic human airways and capable of increasing MUC5AC expression in NHBE cells (38). We found that supplementing EGFR blockade with a neutralizing antibody to TGF- β_2 led to complete attenuation of the MUC5AC response to compressive stress (Figure 5B), and that TGF- β_2 blockade alone was similarly effective (Figure 5C). These results resonate with those of Chu and colleagues (38), and support a role for TGF- β_2 in regulation of MUC5AC expression. Moreover, these findings refocus attention on how TGF- β_2 might be activated by airway epithelial cells. Unlike TGF- β_1 and - β_3 , TGF- β_2 is not a target of $\alpha v \beta 6$ or $\alpha v \beta 8$ integrin mediated activation (45, 46). Notably, previous microarray studies (47) identified the urokinase plasminogen activator and its receptor as targets of mechanical stress in NHBE cells. Urokinase activation by mechanical stress leads to enhanced plasmin generation and MMP-9 activity (47), both of which can initiate proteolytic processing of TGF- β into its active form (45), providing a candidate mechanism linking mechanical stress to TGF- β_2 activation.

Despite the robust nature of changes in MUC5AC protein in response to chronic compressive stress, we were disappointed to find an absence of changes in transcript levels for *MUC5AC* and other epithelial phenotypic markers after 14 days of compressive stress. We did observe a trend toward increasing *MUC5AC* (though not statistically significant, $P = 0.08$), and a proportional and statistically significant decrease in *FOXA2*, an inhibitor of goblet cell differentiation, after 1 day of mechanical stress. The lack of more robust changes in transcript levels frustrated our efforts to characterize the detailed temporal shifts in epithelial phenotypes and gene expression under the influence of compressive stress. One possible explanation for the more muted changes in message levels is that the time points at which we sampled transcripts (Days 1 and 14) were not as informative as other time points within the experimental window. Alternatively, it is possible that the increase in intracellular mucin arose not from increased transcription, but from modifications in secretion (48), which could lead to accumulation of mucin within secretory granules. Another possible explanation is that mechanical stress modified the efficiency of mucin post-translational modifications independent of transcriptional effects, altering protein stability or immunodetection by the anti-MUC5AC antibody. Whatever the mechanism, we did observe significant changes in intracellular MUC5AC protein levels via immunofluorescence, Western blotting, and slot blotting with chronic mechanical stress, emphasizing that measurements at the protein level, especially of proteins stably expressed as a function of commitment to a specialized phenotype, provide an attractive and physiologically relevant outcome measure. In this regard, the stability of the cilia marker β -tubulin IV in the presence of shifting MUC5AC protein levels (Figure 3) suggested that mechanical stress may enhance the mucin secretory phenotype without compensatory loss of ciliated cells.

Clearly additional studies will be needed to elucidate the cellular proliferation, differentiation, and/or transdifferentiation processes involved in the response to compressive stress (19, 49–51), and the molecular mechanisms by which EGFR and TGF- β_2 enhance the MUC5AC-expressing cell population.

In summary, our findings demonstrate that intermittent episodes of compressive stress, similar in magnitude to those occurring during airway constriction, are sufficient to enhance epithelial MUC5AC expression. One consequence of mucin accumulation driven by mechanical compression would be the potential, once secreted, for this accumulated mucin to amplify and prolong airway obstruction. Our observation that EGFR activation and TGF- β_2 signaling both contribute to the response to compressive stress indicates that not only is the previously detailed EGFR response to compressive stress (13) biologically relevant, but also that additional mechanosignaling pathways related to TGF- β_2 remain to be elucidated. In combination with our prior demonstration that key fibrogenic behaviors are driven by the same mechanical stimulus applied to bronchial epithelial cells (39), it is apparent that the mechanical environment present in the constricted airway can stimulate multiple important features of airway remodeling. Thus, prevention or rapid reversal of airway constriction may be an important goal of asthma control that aids in averting or limiting multiple aspects of airway remodeling.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* 2002;109:571–577.
- Perez-Vilar J, Sheehan JK, Randell SH. Making more MUCS. *Am J Respir Cell Mol Biol* 2003;28:267–270.
- Fahy JV. Remodeling of the airway epithelium in asthma. *Am J Respir Crit Care Med* 2001;164:S46–S51.
- Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004;350:2645–2653.
- Cohn L. Mucus in chronic airway diseases: sorting out the sticky details. *J Clin Invest* 2006;116:306–308.
- Ordóñez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, et al. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* 2001;163:517–523.
- Vestbo J. Epidemiological studies in mucus hypersecretion. *Novartis Found Symp* 2002;248:3–12; discussion 12–9, 277–282.
- Williams OW, Sharafkhan A, Kim V, Dickey BF, Evans CM. Airway mucus: from production to secretion. *Am J Respir Cell Mol Biol* 2006;34:527–536.
- Basbaum C, Lemjabbar H, Longphre M, Li D, Gensch E, McNamara N. Control of mucin transcription by diverse injury-induced signaling pathways. *Am J Respir Crit Care Med* 1999;160:S44–S48.
- Rose MC, Nickola TJ, Voynow JA. Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. *Am J Respir Cell Mol Biol* 2001;25:533–537.
- Voynow JA, Gendler SJ, Rose MC. Regulation of mucin genes in chronic inflammatory airway diseases. *Am J Respir Cell Mol Biol* 2006;34:661–665.
- Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;86:245–278.
- Tschumperlin DJ, Dai G, Maly IV, Kikuchi T, Laiho LH, McVittie AK, Haley KJ, Lilly CM, So PT, Lauffenburger DA, et al. Mechanotransduction through growth-factor shedding into the extracellular space. *Nature* 2004;429:83–86.
- Burgel PR, Nadel JA. Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. *Thorax* 2004;59:992–996.
- Casalino-Matsuda SM, Monzon ME, Forteza RM. Epidermal growth factor receptor activation by epidermal growth factor mediates oxidant-induced goblet cell metaplasia in human airway epithelium. *Am J Respir Cell Mol Biol* 2006;34:581–591.
- Guzman K, Randell SH, Nettekheim P. Epidermal growth factor regulates expression of the mucous phenotype of rat tracheal epithelial cells. *Biochem Biophys Res Commun* 1995;217:412–418.
- Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA. Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci USA* 1999;96:3081–3086.
- Takeyama K, Fahy JV, Nadel JA. Relationship of epidermal growth factor receptors to goblet cell production in human bronchi. *Am J Respir Crit Care Med* 2001;163:511–516.
- Tyner JW, Kim EY, Ide K, Pelletier MR, Roswit WT, Morton JD, Battaile JT, Patel AC, Patterson GA, Castro M, et al. Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. *J Clin Invest* 2006;116:309–321.
- Vargaftig BB, Singer M. Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L808–L818.
- Zhen G, Park SW, Nguyen LT, Rodriguez MW, Barbeau R, Paquet AC, Erle DJ. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am J Respir Cell Mol Biol* 2007;36:244–253.
- Kikuchi T, Shively JD, Foley JS, Drazen JM, Tschumperlin DJ. Differentiation-dependent responsiveness of bronchial epithelial cells to IL-4/13 stimulation. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L119–L126.
- Ross AJ, Dailey LA, Brighton LE, Devlin RB. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2007;37:169–185.
- Ressler B, Lee RT, Randell SH, Drazen JM, Kamm RD. Molecular responses of rat tracheal epithelial cells to transmembrane pressure. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1264–L1272.
- Tschumperlin DJ, Shively JD, Swartz MA, Silverman ES, Haley KJ, Raab G, Drazen JM. Bronchial epithelial compression regulates MAP kinase signaling and HB-EGF-like growth factor expression. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L904–L911.
- Wiggs BR, Hrousis CA, Drazen JM, Kamm RD. On the mechanism of mucosal folding in normal and asthmatic airways. *J Appl Physiol* 1997;83:1814–1821.
- Yuan-Chen Wu D, Wu R, Reddy SP, Lee YC, Chang MM. Distinctive epidermal growth factor receptor/extracellular regulated kinase-independent and -dependent signaling pathways in the induction of airway mucin 5B and mucin 5AC expression by phorbol 12-myristate 13-acetate. *Am J Pathol* 2007;170:20–32.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–408.
- Sheehan JK, Howard M, Richardson PS, Longwill T, Thornton DJ. Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug. *Biochem J* 1999;338:507–513.
- Henke MO, Renner A, Huber RM, Seeds MC, Rubin BK. MUC5AC and MUC5B mucins are decreased in cystic fibrosis airway secretions. *Am J Respir Cell Mol Biol* 2004;31:86–91.
- Wan H, Kaestner KH, Ang SL, Ikegami M, Finkelman FD, Stahlman MT, Fulkerson PC, Rothenberg ME, Whitsett JA. Foxa2 regulates alveolarization and goblet cell hyperplasia. *Development* 2004;131:953–964.
- Yoshisue H, Puddicombe SM, Wilson SJ, Haitchi HM, Powell RM, Wilson DI, Pandit A, Berger AE, Davies DE, Holgate ST, et al. Characterization of ciliated bronchial epithelium 1, a ciliated cell-associated gene induced during mucociliary differentiation. *Am J Respir Cell Mol Biol* 2004;31:491–500.
- You Y, Huang T, Richer EJ, Schmidt JE, Zabner J, Borok Z, Brody SL. Role of f-box factor foxj1 in differentiation of ciliated airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L650–L657.

34. Brody SL, Yan XH, Wuerffel MK, Song SK, Shapiro SD. Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. *Am J Respir Cell Mol Biol* 2000;23:45–51.
35. Chen R, Perrone CA, Amos LA, Linck RW. Tektin B1 from ciliary microtubules: primary structure as deduced from the cDNA sequence and comparison with tektin A1. *J Cell Sci* 1993;106:909–918.
36. Gomperts BN, Kim LJ, Flaherty SA, Hackett BP. IL-13 regulates cilia loss and foxj1 expression in human airway epithelium. *Am J Respir Cell Mol Biol* 2007;37:339–346.
37. Yoshisue H, Hasegawa K. Effect of MMP/ADAM inhibitors on goblet cell hyperplasia in cultured human bronchial epithelial cells. *Biosci Biotechnol Biochem* 2004;68:2024–2031.
38. Chu HW, Balzar S, Seedorf GJ, Westcott JY, Trudeau JB, Silkoff P, Wenzel SE. Transforming growth factor-beta2 induces bronchial epithelial mucin expression in asthma. *Am J Pathol* 2004;165:1097–1106.
39. Tschumperlin DJ, Shively JD, Kikuchi T, Drazen JM. Mechanical stress triggers selective release of fibrotic mediators from bronchial epithelium. *Am J Respir Cell Mol Biol* 2003;28:142–149.
40. Tschumperlin DJ, Drazen JM. Chronic effects of mechanical force on airways. *Annu Rev Physiol* 2006;68:563–583.
41. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem* 2003;278:17036–17043.
42. Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L730–L739.
43. Kim KC, Zheng QX, Brody JS. Effect of floating a gel matrix on mucin release in cultured airway epithelial cells. *J Cell Physiol* 1993;156:480–486.
44. Chu EK, Foley JS, Cheng J, Patel AS, Drazen JM, Tschumperlin DJ. Bronchial epithelial compression regulates epidermal growth factor receptor family ligand expression in an autocrine manner. *Am J Respir Cell Mol Biol* 2005;32:373–380.
45. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci* 2003;116:217–224.
46. Neurohr C, Nishimura SL, Sheppard D. Activation of transforming growth factor-beta by the integrin alphavbeta8 delays epithelial wound closure. *Am J Respir Cell Mol Biol* 2006;35:252–259.
47. Chu EK, Cheng J, Foley JS, Mecham BH, Owen CA, Haley KJ, Mariani TJ, Kohane IS, Tschumperlin DJ, Drazen JM. Induction of the plasminogen activator system by mechanical stimulation of human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2006;35:628–638.
48. Button B, Picher M, Boucher RC. Differential effects of cyclic and constant stress on ATP release and mucociliary transport by human airway epithelia. *J Physiol* 2007;580:577–592.
49. Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, DeMayo FJ, Burns AR, Smith C, Reynolds SD, *et al.* Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. *Am J Respir Cell Mol Biol* 2004;31:382–394.
50. Hajj R, Baranek T, Le Naour R, Lesimple P, Puchelle E, Coraux C. Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties. *Stem Cells* 2007;25:139–148.
51. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol* 2004;164:577–588.