Rhythmic Pressure Waves Induce Mucin5AC Expression via an EGFR-Mediated Signaling Pathway in Human Airway Epithelial Cells

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Rhythmic pressure waves (RPW), mimicking the mechanical forces generated during normal breathing, play a key role in airway surface liquid (ASL) homeostasis. As a major component of ASL, we speculated that the mucin5AC (MUC5AC) expression must also be regulated by RPW. However, fewer researches have focused on this question. Therefore, our aim was to test the effect and mechanism of RPW on MUC5AC expression in cultured human bronchial epithelial cells. Compared with the relevant controls, the transcriptional level of MUC5AC and the protein expressions of MUC5AC, the phospho-epidermal growth factor receptor (p-EGFR), phospho-extracellular signal-related kinase (p-ERK), and phospho-Akt (p-Akt) were all significantly increased after mechanical stimulation. However, this effect could be significantly attenuated by transfecting with EGFR-siRNA. Similarly, pretreating with the inhibitor of ERK or phosphatidylinositol 3-kinases (PI3K)/Akt separately or jointly also significantly reduced MUC5AC expression. Collectively, these results indicate that RPW modulate MUC5AC expression via the EGFR-PI3K-Akt/ERK-signaling pathway in human bronchial epithelial cells.

Introduction

MUCUS CLEARANCE IS an essential, innate lung defense in the airways, and efficient clearance requires the coordinated interaction of two layers, an overlying mucus layer, which binds and transports various inhaled materials, and an underlying layer, which bathes the cilia that comprise the airway surface liquid (ASL). It has been widely assumed that the hydration status of the ASL, ciliary activity, and glycosylated mucin was the dominant determinant of mucus clearance (Matsui *et al.*, 1998; Randell and Boucher, 2006; Button and Boucher, 2008). It has become increasingly clear that the phasic motion of the airway wall during normal tidal breathing (in our present study termed rhythmic pressure waves [RPW]) regulates the hydration status of ASL and ciliary activity via nucleotides and nucleosides (Lazarowski *et al.*, 2004; Tarran *et al.*, 2005; Button *et al.*, 2007; Mall, 2008). Considering all of the data, mucin5AC (MUC5AC), the prominent glycosylated mucins of the mucus layer that assists in the clearance of inhaled foreign bodies (Morcillo and Cortijo, 2006), must be under the regulation of RPW as well. Unfortunately, fewer studies have investigated the physical effects and molecular mechanisms by which RPW effect MUC5AC expression in human bronchial epithelial cells.

RPW, generated during normal tidal breathing throughout life, consist of two main stresses: airflow-induced rhythmic shear stress and transepithelial pressure gradient-induced rhythmic compressive stress (Wirtz and Dobbs, 2000; Button and Boucher, 2008). Tschumperlin and colleagues have previously reported that mechanical stimulation, both in the intact mouse and in cell culture, can activate the epidermal growth factor receptor (EGFR) by releasing its ligands from the basolateral compartment of bronchial epithelial cells (Tschumperlin *et al.*, 2002, 2004; Tschumperlin, 2004). In addition, the EGFR is a central mediator of MUC5AC expression through a complex signaling cascade under both physiological and pathological conditions (Takeyama *et al.*, 2001a; Shao *et al.*, 2003; Li *et al.*, 2012a, 2012b). Therefore, we hypothesized that RPW induce MUC5AC expression via an EGFR-signaling pathway.

Moreover, many studies have previously indicated that RPW selectively activate extracellular signal-related kinase (ERK), phosphatidylinositol 3-kinases (PI3K), and Akt (Tschumperlin *et al.*, 2002; Miyahara *et al.*, 2007; Shiomi *et al.*, 2011). ERK and PI3K/Akt are critical mediators of the induction of MUC5AC expression (Hewson *et al.*, 2004; Song *et al.*, 2005; Kim *et al.*, 2009). Therefore, we evaluated whether ERK and PI3K/Akt participate in RPW-induced MUC5AC expression. In the present study, we aimed to examine the

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hypothesis that RPW, the primary mechanical forces during normal tidal breathing, might modulate the appropriate expression of MUC5AC via an EGFR-PI3K-Akt/ERK MAPK/nitric oxide signaling cascade.

Materials and Methods

Materials

The bronchial epithelial growth medium (BEGM) Bullet Kit was purchased from Clonetics. Fetal bovine serum (FBS) was purchased from Gibco. The mouse monoclonal antibody to MUC5AC (45M1) was purchased from Neomarker. Anti-phospho-EGFR (p-EGFR), anti-phospho-ERK (p-ERK), anti-phospho-Akt (p-Akt), anti-EGFR, anti-ERK, anti-Akt, and anti- β -actin antibodies were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jinqiao Biotech. Inhibitors to ERK1/2 (PD-98059) and PI3K/Akt (LY-294002) were purchased from Sigma-Aldrich.

Cell culture

Human bronchial epithelial cells 16HBE (1×10^5 /mL), kindly provided by the Experimental Medical Research Center of Guangzhou Medical College (Guangzhou, Guangdong, China), were seeded on a permeable support (12 mm in diameter, 0.4 μ m pore size; Transwell-clear, Costar; Corning, Inc.) at an air-liquid interface (ALI) in the BEGM Bullet Kit supplemented with 0.1% insulin, 0.1% triiodothyronine, 0.1% hydrocortisone, 0.1% retinoic acid, 0.1% epinephrine, 0.1% transferrin, 0.4% bovine pituitary extract, 10% FBS, penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a well-humidified (>95%) atmosphere of 5% CO₂ at 37°C. The cells were passaged when 80%–90% confluence was reached. Before the experiments, the cells were removed to the same medium without FBS (a serum-free medium) for another 24 h to maintain the basal levels of MUC5AC production.

RPW devices

To imitate the airflow-induced rhythmic shear stress that occurs *in vivo*, four cultures were placed in a six-well plate on a controlled rotary device, rotating the plate in a go/stop manner, which resulted in an acceleration/deceleration phenomenon because of inertia. The shear stress over the apical surface caused by the relative velocities between ASL and the underlying epithelia are similar to airflow-induced shear stress during breathing (Tarran *et al.*, 2005; Even-Tzur *et al.*, 2006). The rates of acceleration/deceleration were ordered at 120 ms (0.5 dynes/cm², note that airway wall shear stress are constant on 0.5 dynes/cm² across all of the airway generations) and at 40 cycles/min (note that normal tidal breathing has both inspiratory and expiratory airflows, so for 20 breaths/min, there are 40 cycles/min of shear stress.) (Tarran *et al.*, 2005; Button and Boucher, 2008). Control cells were also placed in the device without rotating.

To imitate transepithelial pressure gradient-induced rhythmic compressive stress, each culture was fitted with a silicon plug with two ports. The input port connected with a constant continuous pressure reservoir is used to deliver a 5% CO₂ pressure cylinder via a humidified chamber maintained at 37°C into the culture, and the output port connected with the outside is used to balance atmospheric

pressure. For rhythmic compressive stress, the system's microprocessor controls the operation of a pair of high-speed microsolenoid valves, which are located on both silicon plugs of the input port and the output port to alternate the pressure in the culture between chamber pressure (8.5 cmH₂O for 1 s in the sealed state) and atmospheric pressure (0 cmH₂O for 2 s in the open state), at a frequency of 20 cycles/min (Button *et al.*, 2007, 2008). Control cells were treated similarly by being placed in the device, but were exposed at 0 cmH₂O without rhythmic pressure change.

Protocol

MUC5AC mRNA expression was examined over the course of an 8-h application of RPW by real-time polymerase chain reaction (PCR). Total RNA were collected at 30-min intervals up to 4 h and again at 8 h during the continuous stimulation period. The control cells were sampled at the same time points without mechanical stimulation. Concurrently, total protein was also extracted at the above time points, and MUC5AC protein expression was measured by using enzyme-linked immunosorbent assay (ELISA). MUC5AC protein expression immediately before mechanical stimulation was served as a control. The time of peak expression of MUC5AC expression was then determined.

Inhibitor study

To investigate the signaling pathway, cells were pretreated with the inhibitor of ERK (PD-98059, 50 μ M, 30 min) or PI3K/Akt (LY-294002, 50 μ M, 30 min) (Atherton *et al.*, 2003; Sung *et al.*, 2011) separately or jointly before they were exposed to RPW. Inhibitor controls were pretreated with these inhibitors without mechanical stimulation, and negative controls were treated with equivalent amounts of dimethyl sulfoxide at the same time.

Real-time PCR

Total RNA was isolated using the Trizol solution (Takara) and 2 μ g of total RNA was used for synthesizing complementary DNA (cDNA) using the iScript complementary DNA synthesis kit (Takara). Twenty nanograms of cDNA was used for real-time PCR in the mixture of primers and the iQ SYBR Green supermix in an iCycler (Takara). The following primers were used: MUC5AC, forward 5'-CACTC TACCACTCCCTGCTTC-3' and reverse 5'-CTTGACTAACCTCTTGACCAC-3'; EGFR, forward 5'-GGCTCACCCTCCA GAAGCTT-3' and reverse 5'-TGCCTCTCTTGCCGGAAT-3'. The PCR mixture was denatured at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. GAPDH was used as an internal control. Relative levels were determined by the $\Delta\Delta$ Ct method.

siRNA transfection

Two different siRNAs targeting EGFR (EGFR1-siRNA and EGFR2-siRNA) and control nontargeting (NT-siRNA) were synthesized by Sangong. The sequences of the siRNAs are as follows: EGFR1-siRNA, 5'-GGAGCUGCCCAUGAGAAU (dTdT)-3'; EGFR2-siRNA, 5'-AUUUCUCAUGGGCAGCUCC (dTdT)-3'; and NT-siRNA, 5'-GCGCGCUUUGUAGGAUUCG (dTdT)-3'. Each siRNA was mixed with 1 μ L of Lipofectamine 2000 (Invitrogen) and diluted with Opti-MEM (Invitrogen)

to 100 μ L. Twenty minutes later, the RNA-lipid complexes at a final volume of 400 μ L were added to each well and incubated for an additional 48 or 72 h before mechanical stimulation. The knockdown of the EGFR was estimated by real-time PCR and Western blotting.

ELISA experiment

After various treatments, the amount of MUC5AC protein was measured using the specific ELISA kit (Yuanye) according to the manufacturer's instructions. Briefly, cells were lysed with an ice-cold lysis buffer containing protease inhibitor cocktail, and the total protein in the cell lysates was measured by using the Bradford assay. After appropriate dilution, each sample (50 μ L) was incubated with the bicarbonate-carbonate buffer (50 μ L) in a 96-well plate at 40°C overnight. After blocking with 2% bovine serum albumin (BSA), the samples were incubated with 50 μ L of the mouse monoclonal MUC5AC antibody (1:100) at 37°C for 1 h followed by 100 μ L of the HRP-conjugated goat anti-mouse IgG (1:5 000) at room temperature for 1 h. The color reaction was developed with a 3,3',5,5'-tetramethylbenzidine peroxidase solution and terminated with H₂SO₄. Absorbance was read at 450 nm using a microplate reader (Sunrise remote; Tecan). The amount of MUC5AC protein in each sample was normalized to the total protein in the lysates and was expressed as μ g/mg lysates protein.

Western blotting

The phosphorylation of EGFR, ERK1/2, and Akt proteins were detected by Western blotting. In brief, cells were lysed with the lysis buffer containing protease inhibitor cocktail on the ice after various treatment. The total protein in the cell lysates was measured by using the Bradford assay. Equal amounts of the lysates (20 μ L) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% BSA, the PVDF membranes were probed with appropriate primary antibodies at 4°C overnight followed by secondary antibodies conjugated with HRP at 37°C for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence. The relative amount of the protein of interest was normalized to the amount of its total protein or β -actin. Densitometric quantification of bands was performed with Quantity One software (Bio-Rad).

Statistical analysis

Values are presented as the mean $\pm$ SD. All experiments were repeated three times using six replicate wells in each independent experiment. Data were analyzed using the SPSS 10.0 statistical package (SPSS, Inc.). Differences were evaluated for statistical significance using the Student's *t*-test for paired comparison or one-way ANOVA for multiple comparisons followed by the Bonferroni's *post hoc* test after normality with the W test. *p*-Values of less than 0.05 were considered statistically significant.

Results

RPW increase the mRNA and protein expressions of MUC5AC

As shown in Figure 1, continuous RPW applied for 8 h caused increased MUC5AC mRNA expression beginning at

FIG. 1. The effect of RPW on the expressions of MUC5AC mRNA and protein in 16HBE cells. MUC5AC mRNA and protein were assessed by real-time PCR and ELISA at the indicated time points during the continuous RPW stimulation, respectively. The data are present as the mean $\pm$ SD ($n=6$). # $p < 0.05$ and * $p < 0.01$ compared with the control cells without mechanical stimulation. MUC5AC, mucin5AC; RPW, rhythmic pressure waves; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

1 h, reached a plateau at 1.5 h after the exposure, and then varying little during the ensuing period. However, MUC5AC protein expression reached the plateau after 2 h of continuous RPW. The plateau time point was slightly earlier in stress-induced MUC5AC mRNA expression (1.5 h) than that in MUC5AC protein expression in response to continuous mechanical stimulation (2 h), which was evaluated as the delayed production in protein compared with mRNA. Since we mainly aimed at the expression of the MUC5AC protein after applying mechanical stress, the plateau time point of 2 h was selected as the optimal response time in ensuing experiments. Taken together, continuous application of rhythmic pressure increases mRNA and protein expression of MUC5AC.

RPW increase the phosphorylation of EGFR, ERK1/2, and Akt

To test the effect of RPW on the p-EGFR, we measured total and phosphorylated EGFR expressions following exposure to RPW for 2 h using Western blotting. Compared with the static group, p-EGFR increased significantly in response to RPW ($p < 0.01$) (Fig. 2A). Meanwhile, similar increases in the protein expressions of p-ERK1/2 and p-Akt in response to RPW were also observed ($p < 0.01$) (Fig. 2B, C), indicating that rhythmic pressure may activate EGFR, ERK1/2, and Akt.

The EGFR may mediate RPW-induced MUC5AC protein expression

We have reported that RPW can increase both p-EGFR and MUC5AC expressions, and thus, to prove the involvement of EGFR in RPW-induced MUC5AC expression, siRNA experiments were performed. As Figure 3A shows, the two siRNAs targeted for EGFR significantly suppressed endogenous levels of EGFR mRNA and protein in transfected cells compared with the control NT-siRNA cells ($p < 0.01$). Since the two siRNAs showed similar results, we performed the subsequent studies of the effects of EGFR reduction on RPW-

FIG. 2. The effect of RPW on the activation of EGFR, ERK1/2, and Akt in 16HBE cells. Cell lysates were prepared after 2 h of RPW exposure; p-EGFR (A), p-ERK1/2 (B), and p-Akt (C) protein expressions were measured by Western blotting analyses using specific antibodies. The intensity of the blots was normalized to its total protein expression (t-EGFR, t-ERK1/2, and t-Akt). The data are present as the mean $\pm$ SD ($n=6$). * $p < 0.01$ compared with the control cells under static condition. EGFR, epidermal growth factor receptor; p-EGFR, phospho-epidermal growth factor receptor; p-ERK, phospho-extracellular signal-related kinase.

induced cellular responses using a siRNA pool derived by combining the two EGFR targeted sequences. Under the condition in which the EGFR expression was reduced, RPW-induced MUC5AC protein expression was significantly attenuated ($p < 0.01$) (Fig. 3B), yet the baseline MUC5AC protein expression was rarely affected, indicating that EGFR activated by RPW is involved, at least in part, in RPW-induced MUC5AC protein expression in 16HBE cells.

The EGFR controls the activation of the ERK1/2 and Akt pathways

Since we have demonstrated that RPW activate EGFR, ERK1/2, and Akt, and it is known that the activation of ERK1/2 and Akt pathways were regulated by EGFR activation, we address whether RPW activate ERK1/2 and Akt pathways via the EGFR. Figure 4 shows that EGFR reduction by its specific siRNA significantly inhibits the activation of ERK1/2 and Akt due to RPW ($p < 0.01$), suggesting that the activation of EGFR induces the phosphorylation of ERK1/2 and Akt in 16HBE cells.

ERK1/2 and Akt contribute to MUC5AC expression in response to rhythmic pressure

It is clear that ERK is a key intermediate in the EGFR-dependent MUC5AC pathway (Atherton *et al.*, 2003). Therefore, we hypothesized that ERK was a critical downstream molecular component of the EGFR-dependent path-

FIG. 3. The role of EGFR in RPW-induced MUC5AC protein expression. 16HBE cells were transfected with a siNT or specific siRNA for EGFR (siEGFR1, siEGFR2, or siEGFR) before applying RPW. After transfection, the expressions of EGFR mRNA and protein (A) were measured by real-time PCR and Western blotting, respectively. The expression of MUC5AC was detected by ELISA after 2 h of RPW exposure (B). The data are present as the mean $\pm$ SD ($n=6$). * $p < 0.01$ and # $p < 0.05$ compared with the siNT-transfected static cells, ^ $p < 0.01$ compared with the siNT-transfected RPW cells. siNT, nontargeting siRNA.

way and can play an important role in rhythmic pressure-induced MUC5AC expression. Meanwhile, previous studies indicated that the Akt pathway may be activated during high-volume ventilation (Miyahara *et al.*, 2007). Therefore, to test these molecules that function downstream of EGFR-dependent rhythmic pressure-induced MUC5AC expression, we pretreated with an ERK-specific inhibitor PD-98059 or a PI3K/Akt-specific inhibitor LY-294002. Both of these inhibitors completely blocked the increases in p-ERK and p-Akt (Fig. 5A, B). Meanwhile, pretreating with inhibitor(s) separately or jointly significantly attenuated increased MUC5AC protein expression in response to RPW, and the inhibition effect of the combination of inhibitors was slightly greater in RPW-induced MUC5AC protein expression than the inhibitor alone (Fig. 5C). As a result, we speculated that ERK- and PI3K/Akt-signaling pathways are important for EGFR-dependent rhythmic pressure-induced MUC5AC expression, paralleling the role of the EGFR in mediating rhythmic pressure-induced MUC5AC expression.

Discussion

As an open organ, the lung is subjected to direct contact with various substances during breathing, such as inhaled

FIG. 4. The role of EGFR in RPW induced the phosphorylation of ERK1/2 and Akt. The cells were transfected with a siNT or specific siRNA for EGFR (siEGFR) before applying RPW. Cell lysates were prepared after 2 h of RPW exposure; p-ERK1/2 (**A**) and p-Akt (**B**) protein expressions were measured by Western blotting analyses using specific antibodies. The intensity of the blots was normalized to its total protein expression (t-ERK1/2 and t-Akt). Data are present as the mean $\pm$ SD ($n=6$). * $p<0.01$ and # $p<0.05$ compared with the siNT-transfected static cells, ^ $p<0.01$ compared with the siNT-transfected RPW cells.

viruses, bacteria, and other noxious particles. The appropriate clearance of mucus is of particular importance (Randall and Boucher, 2006; Button and Boucher, 2008; Smith *et al.*, 2008). While much is known about physiologically relevant mechanical stresses, in this study termed RPW, are necessary for maintaining proper rheological properties of ASL, effective cilia beats, and proper mucus clearance. It is clear that the rate of mucus clearance directly depends on an effective cilia beat, hydration status, and mucin production of ASL, which alters viscoelasticity. Studies have previously indicated that rhythmic pressure plays a key role in regulating the hydration status of ASL by the release of 5' nucleotide triphosphates (ATP and UTP), along with its metabolite adenosine, via purinergic receptor (P2Y₂, P2Y₆, and A_{2b} receptor)-dependent mechanisms. It also regulates the cilia beat frequency by increasing the cellular calcium concentration via ATP-mediated A_{2b} purinoceptor activation (Lazarowski *et al.*, 2004; Tarran *et al.*, 2005; Button *et al.*, 2007; Mall, 2008). Combined with active ions, liquid, and cilia in an identical condition, as another essential component of ASL, mucin must be under the regulation of rhythmic pressure as well.

RPW generated during normal tidal breathing consist of two essential components, shear stress and compressive stress. Shear stress is induced by airflow moving across the airway surface epithelia, and this stress force peaks at 0.5 dyne/cm² during normal tidal breathing (Lazarowski *et al.*, 2004; Button and Boucher, 2008). Compressive stress is induced by transepithelial pressure gradients, approaching 8.5 cmH₂O during normal tidal breathing, 20 cmH₂O during forced expiration, and even exceeding 30 cmH₂O during bronchoconstriction (Ressler *et al.*, 2000; Button *et al.*, 2007, 2008). During coughing, mechanical forces on airways are even more extreme that the level of shear stress can rapidly reach as high as 1 700 dyne/cm², and compressive stress 200 cmH₂O. It is known that increased MUC5AC expression

FIG. 5. The effect of a specific inhibitor of ERK or PI3K/Akt on RPW induced the activation of ERK1/2 and Akt, and the expression of the MUC5AC protein. The cells were pretreated with the ERK inhibitor PD98059, PI3K/Akt inhibitor LY294002, or an equivalent amount of DMSO (control group) 30 min before applying RPW. Cell lysates were prepared after 2 h of RPW exposure; p-ERK1/2 (**A**) and p-Akt (**B**) protein expressions were measured by Western blotting analyses using specific antibodies. The intensity of the blots was normalized to its total protein expression (t-ERK1/2 and t-Akt). The MUC5AC protein expression (**C**) was detected by ELISA using antibodies directed against MUC5AC. Data are present as the mean $\pm$ SD ($n=6$). * $p<0.01$ and ** $p<0.05$ compared with the DMSO-treated static group, # $p<0.01$ and ## $p<0.05$ compared with the DMSO-treated RPW group, ▲ $p<0.05$ compared with the separate inhibitor-treated RPW group. PI3K, phosphatidylinositol 3-kinases; DMSO, dimethyl sulfoxide.

represents a common response to the higher magnitude forces that are present during bronchoconstriction in pathological conditions (Tschumperlin *et al.*, 2004; Park and Tschumperlin, 2009). In the present study, we aimed to test whether RPW generated during normal tidal breathing could induce MUC5AC expression under physiological conditions. Therefore, we continuously applied physiological levels of mechanical force on 16HBE for 8 h. Sampled at set time points, we found that MUC5AC protein expression rapidly increased in 16HBE in response to rhythmic pressure and continued to increase its expression until arriving at a plateau of expression at 2 h (Fig. 1). This trend is similar, but much lower, than previous reports (Park and Tschumperlin, 2009). These results indicate that not only high-magnitude mechanical forces generated in pathological conditions, but also rhythmic pressure generated during normal tidal breathing can increase MUC5AC expression and eventually

influence the mucin components of the mucus layer, and ultimately, mucus clearance.

We further sought to explore the key signaling molecules in this process. It has long been known that the EGFR is involved in multiple processes in airway biology, including MUC5AC expression, goblet cell hyperplasia, airway wall remodeling, and epithelial repair (Davies *et al.*, 1999; Takeyama *et al.*, 1999, 2001b). Some studies have determined that transepithelial pressure gradients (30 cmH₂O) generated during bronchoconstriction separate the lateral membranes of adjacent cells causing an increased concentration of EGFR ligands, including heparin-binding epidermal growth factor-like growth factor (HB-EGF), epiregulin, and amphiregulin, but not transforming growth factor (TGF)- α , and subsequent signaling (Takeyama *et al.*, 2001b; Tschumperlin *et al.*, 2002; Chu *et al.*, 2005; Park and Tschumperlin, 2009). Therefore, we wondered whether the EGFR could be activated by rhythmic pressure under physiological conditions. Our results indicated that phosphorylated EGFR expression increased significantly in response to rhythmic pressure. As a result, we continued to study the effect of inhibiting rhythmic pressure-induced EGFR activation using siRNA-EGFR. We found that RPW-induced MUC5AC expression was significantly attenuated. Together, these data indicate that the EGFR is a crucial, but not the sole regulator of rhythmic pressure-induced MUC5AC expression.

Next, we continued to explore the downstream signaling molecules responsible for RPW-induced EGFR activation. It has been previously indicated that mechanical forces generated during bronchoconstriction could enhance the phosphorylation of ERK in 16HBE (Tschumperlin *et al.*, 2002; An *et al.*, 2009), and we previously found that inhibition of EGFR transduction with EGFR-siRNA attenuated the phosphorylation of ERK and the expression of MUC5AC in response to rhythmic pressure. Therefore, we tested whether ERK could be phosphorylated by RPW through an EGFR-dependent pathway under physiological conditions with PD98059, a specific inhibitor of ERK. We found that the effect of rhythmic pressure on MUC5AC expression was decreased, suggesting that ERK participates in modulating rhythmic pressure-induced MUC5AC expression via EGFR-dependent pathways.

Given that the attenuation with an ERK inhibitor was incomplete, we considered the possibility that PI3K/Akt-signaling cascades might be an additional pathway that regulates rhythmic pressure-induced MUC5AC expression based on the previous demonstration of PI3K/Akt activation in response to high ventilation in lung injury (García-Cardena *et al.*, 2000; Miyahara *et al.*, 2007). It is clear that PI3K/Akt is capable of regulating MUC5AC expression via NF-kappa B activation in airway epithelial cells. Therefore, we hypothesized that PI3K/Akt may be another downstream transducer of EGFR activation. Unsurprisingly, we found that PI3K/Akt blockade with a specific inhibitor to PI3K/Akt inhibited expression of MUC5AC to the same extent that ERK blockade did. Strikingly, the combination of the ERK inhibitor and PI3K/Akt inhibitor resulted in a greater attenuation of the increase in mechanical stimulation-induced MUC5AC protein expression than the inhibitor alone did, indicating a parallel signaling pathway through ERK and Akt resulting in EGFR activation participated rhythmic pressure-induced MUC5AC expression.

Conclusion

Our findings indicate that RPW generated during normal tidal breathing can induce MUC5AC expression. The EGFR mediates this process induced by rhythmic pressure via the ERK- and PI3K/Akt-signaling cascades. The EGFR is a crucial, but not sole, regulator of this process and our understanding of the mechanisms involved in rhythmic pressure-induced MUC5AC expression is far from complete. In the present study, we explored the effect of rhythmic pressure on MUC5AC expression, combining prior research about the regulation of rhythmic pressure on ASL hydration status and cilia beat frequency. Together, these results suggest that the rhythmic pressure generated during normal tidal breathing is important for maintaining ASL homeostasis.

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Disclosure Statement

No competing financial interests exist.

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