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Mechanophysical Stimulations of Mucin Secretion in Cultures of Nasal Epithelial Cells

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ABSTRACT Nasal epithelial cells secret mucins and are exposed in vivo to airflow-induced mechanophysical stresses, including wall shear stress (WSS), temperature, and humidity. In this work, human nasal epithelial cells cultured under air-liquid interface conditions were subjected to fields of airflow-induced oscillatory WSS at different temperature and humidity conditions. Changes in mucin secretion due to WSS were measured and the role of the cytoskeleton in mucin secretion was explored. Mucin secretion significantly increased in response to WSS in a magnitude-dependent manner with respect to static cultures and independently of the airflow temperature and humidity. In static cultures, mucin secretion decreased at high humidity with or without elevation of the temperature with respect to cultures at a comfortable climate. In cultures exposed to WSS, mucin secretion increased at high temperature with respect to cultures at comfortable climate conditions. The polymerization of actin microfilaments was shown to increase mucin secretion under WSS, whereas the dynamics of microtubule polymerization did not affect secretion. In conclusion, the data in this study show that mucin secretion is sensitive to oscillatory WSS as well as high temperature and humidity conditions.

INTRODUCTION

Nasal epithelial goblet cells secrete mucins as an integral part of their function. Second to water, mucins are the primary component of the mucus layer (1), which traps airborne particles for removal from the body through mucociliary clearance. Mucus also has a key role in nasal heating and humidification of the inspired air for the purpose of efficient gas exchange in the lungs and optimal mucociliary clearance (2), as well as in the protection of nasal epithelial cells (NECs) from airflow-induced dehydration. Mucins are high-molecular-weight glycoproteins. Their high carbohydrate content contributes to their great water-holding capacity (3). This dense sugar coating is also responsible for the adhesiveness of the mucus layer (4), which is essential for particle entrapment. Thus, appropriate and sufficient mucin secretion, and controlled regulation of this secretion are crucial for fundamental nasal functions.

NECs are exposed in vivo to different fields of airflow-induced mechanophysical stresses, including wall shear stress (WSS), temperature, and humidity. Computational models of quiet breathing in the healthy nose predicted WSS values as high as 1.6 Pa (5). Because WSSs are linearly related to the local air speed, these values may significantly increase as breathing efforts increase. In addition, the unsteadiness of nasal airflow results in significant variations of WSS with time (i.e., 0.5 Pa/s) even during quiet breathing (6).

To date, the effects of WSS on cellular processes have mostly been explored in endothelial cells. Studies revealed an association of WSS with changes in cell proliferation, cytoskeleton arrangement, and intracellular calcium level, as well as in mechanotransduction mechanisms involving ion channels, integrins, and plasma membrane receptors (7,8). Moreover, mechanical stimulations and specifically WSS have been shown to induce secretion of materials such as surfactant, growth factors, collagens, and ATP from different cell types (9). Although mucin secretion has been studied extensively in response to numerous biochemical stimuli (10–12), the response to mechanical stresses has been explored only for lower-airway cells or tissues (13,14). Recently, we showed that mucin secretion from NEC cultures exposed to steady WSS of 0.1 and 1.0 dyne/cm^2 for >15 min significantly increased in comparison with unstressed cells (15).

The functional response of NECs to different levels of environmental temperature and humidity has been studied only in vivo. One study reported that nasal secretions were inhibited in patients with seasonal allergic rhinitis when exposed by masks to hot humid air (i.e., 37°C and >95% relative humidity (RH) versus 23°C and <20% RH) (16). In another study, cold-air-induced secretion was inhibited by muscarinic antagonist, suggesting that a neural mechanism controls airway responsiveness to environmental stimuli (17). In dogs and rats, extreme environmental temperature and humidity levels were associated with decreased number of goblet cells, epithelial cell infiltration, loss of cilia, and changes in ciliary beat frequency (18–20).

The magnitude of WSS in the nose is similar to that of normal uniform regions of large arteries, where it affects many cellular processes. Rapid mucin secretion from the

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nasal epithelium is essential for the defense response against acute stimulations (21). Our main objective in this work was to explore changes in mucin secretion in cultures of human NECs in response to oscillatory WSS and different temperature and humidity conditions. For this purpose we cultured human NECs under air-liquid interface (ALI) conditions that mimic the in vivo conditions experienced by these cells. In addition, we explored the role of cytoskeleton dynamics in mucin secretion under WSS by disrupting the polymerization of actin microfilaments (MFs) and microtubules (MTs) before exposing the cells to WSS.

MATERIALS AND METHODS

Cell isolation and culture

Human NECs were isolated from nasal turbinates of adult patients who underwent elective endonasal surgical interventions. The use of dissected turbinates was approved by the ethics committee of the Sheba Medical Center at Tel Hashomer (approval No. 2788/2002), and all patients provided written informed consent. The NECs were isolated and cultured in custom-designed wells under ALI conditions as previously described (15,22,23).

Experimental setup

The experimental setup used in this work is described in detail elsewhere (24). Briefly, the system consisted of three parts: 1), an airflow generator (25); 2), a flow chamber with cultured cells (15,22); and 3), a climate chamber (24). The climate chamber was connected to the airflow generator, which drove air under controlled conditions and desired flow profiles in and out of the climate chamber via the flow chamber and over the surface of the cultured cells. The entire tubing in the system was insulated. We computed the uniform field of WSS applied on the cells from the inlet airflow rate using computational models (15).

Experiments with oscillatory WSS under controlled climate conditions

We performed oscillatory sinusoidal WSS experiments under controlled climate conditions using the WSS waveform:

$$\tau(t) = \tau_{\max} \sin \frac{2\pi t}{T_p}, \quad (1)$$

where $\tau_{\max}$ is the wave amplitude and T_p is the period. In this study, $\tau_{\max}$ was either 0.5 or 5.0 dyne/cm^2 and $T_p = 4$ s, which corresponds to quiet breathing at a frequency of 15 breaths per minute. The cells were exposed to oscillatory WSS for a period of 15 min, which under steady WSS yielded significant changes in mucin secretion (15). The climate conditions tested in this work were 25–40°C and 40–80% RH. Exposure to WSS began after the desired temperature and humidity conditions within the climate chambers and the airflow tubing were stabilized. Static cultures for comparison were mounted inside a different climate chamber at the same temperature and humidity levels as in the flow experiments.

All experiments were performed with passage 2, well-differentiated, 10- to 14-day-old ALI cultures of human NECs that were seeded on polytetrafluoroethylene synthetic membranes in the custom-designed wells. We verified the differentiation state of the cultures by staining and imaging intracellular mucins in epithelial goblet cells and visualizing ciliated cells in the culture using scanning electron microscopy. All experiments were performed at least three times, each time with cells from a different subject. The flow chamber was mounted with three well bottoms so that triplicate cultures were always considered.

Exploration of the role of the cytoskeleton in mucin secretion under WSS

To explore the functional role of the actin MFs and the MTs in mucin secretion under WSS, we treated the cultured NECs with different agents that stabilize or inhibit polymerization of the cytoskeleton components. To inhibit actin MFs polymerization, we pretreated the cells immediately before each experiment with Latrunculin B (1 μM, 30 min). Depolymerization of F-actin was prevented by the actin stabilizing agent Phalloidin (1 μM, 60 min), as previously described for airway epithelial cells (26,27). MT polymerization was inhibited by colchicine (5 μM, 60 min) and MTs were stabilized by Taxol (10 nM, 90 min), as previously described for airway epithelial cells (28). These experiments were performed with flow parameters of $\tau_{\max} = 5.0$ dyne/cm^2 and $T_p = 4$ s, and climate conditions of 25°C and 40% RH. Untreated cultures were subjected to the same flow and climate conditions for comparison. Static treated and untreated cultures were mounted in a climate chamber for 15 min under the same temperature and humidity conditions.

Quantification of mucin secretion

For quantification of mucin secretion, all cultures were rinsed 24 h before the experiments and the accumulated mucus was removed from the cultures' apical surface. Twenty-four hours later, just before the beginning of the experiment, we collected the apical 24-h secretion from each culture and measured the mucin concentration to determine the baseline concentration with respect to which the mucin secretion results were normalized (hereafter termed normalized mucin secretion). For the results shown in Figs. 1–3, we performed further processing by calculating the percentages of the normalized mucin secretion data out of the normalized secretion from specific reference conditions, as detailed for each experiment in the Results section below and in the figure captions (and indicated by the *white columns* in the figures). We quantified mucin secretion onto the apical surface of the NEC cultures immediately and 24 h after termination of the experiments using an enzyme-linked lectinosorbent assay that measured the reactivity of glycoproteins equivalent to MUC5B as described previously (15). The MUC5B mucin standards were generously provided by Prof. C. W. Davis (Cystic Fibrosis Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, NC).

Data and statistical analyses

The mucin secretion results are presented as the mean ± standard deviation (SD) from at least three different experiments, each with cells from a different subject. The data in Figs. 1–3 represent percentages of normalized mucin secretion out of normalized secretion from cultures at reference conditions as explained above and indicated by the white columns in the figures. The data in Figs. 4 and 5 represent the normalized mucin secretion without further processing. Statistical analyses were performed with the use of SPSS Release 11.5.1 software. A two-tailed, independent-samples t-test was used to determine statistical significance between two groups. A one-sample t-test was used to compare the sample mean of one data group with a constant value. One-way analysis of variance followed by a Tukey test was used to determine statistical significance between three or more data sets. Data were deemed statistically significant at $p < 0.05$.

RESULTS

Mucin secretion in response to oscillatory WSS at comfortable climate

Mucin secretion measurements performed immediately and 24 h after exposure of NECs to oscillatory WSS at a reference climate with comfortable climate conditions of 25°C

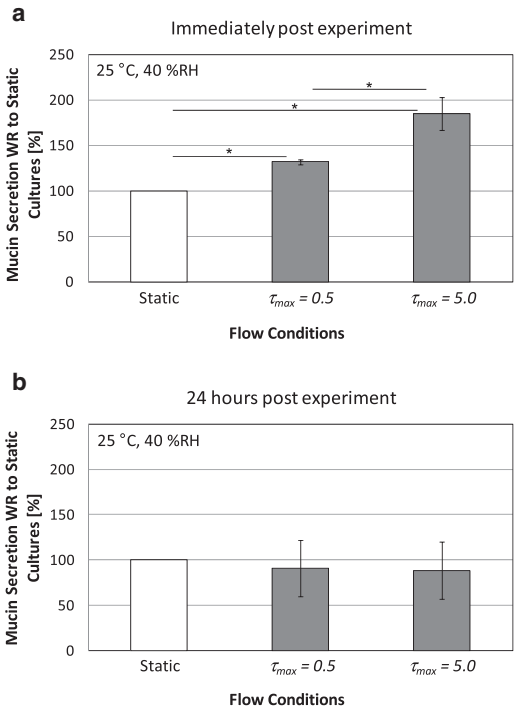

FIGURE 1 Mucin secretion measured (*a*) immediately and (*b*) 24 h after exposure of cultured NECs to oscillatory WSS with $\tau_{max} = 0.5$ and 5.0 dyne/cm^2 and $T_p = 4$ s, for 15 min at 25°C and 40% RH. The results are expressed as the mean ± SD of the percentage of normalized mucin secretion with respect (WR) to normalized secretion from static cultures at 25°C and 40% RH (*white columns*). *$p < 0.05$ between the marked columns.

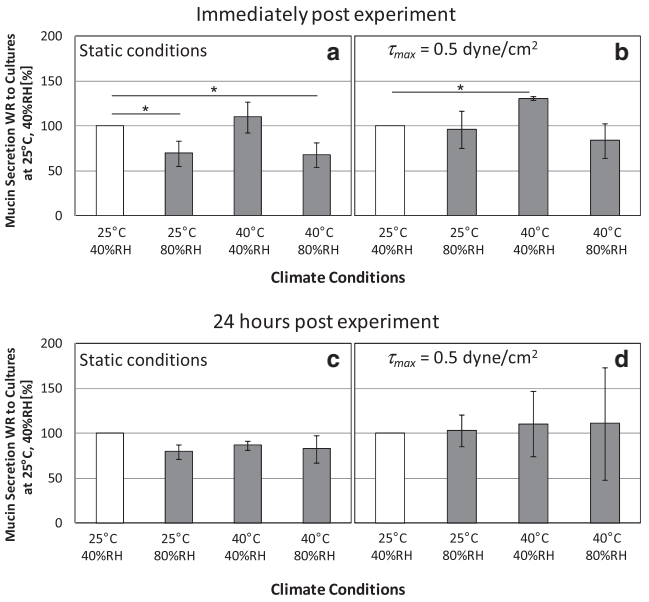

FIGURE 2 Mucin secretion measured (*a* and *b*) immediately and (*c* and *d*) 24 h after exposure of NEC cultures to different climate conditions for 15 min. (*a* and *c*) Static cultures. (*b* and *d*) Cultures exposed to oscillatory sinusoidal WSS with $\tau_{max} = 0.5$ dyne/cm^2 and $T_p = 4$ s. The results are expressed as the mean ± SD of the percentage of normalized mucin secretion from cultures that were exposed to different climate conditions with respect (WR) to normalized mucin secretion from cultures at 25°C and 40% RH (*white columns*). *$p < 0.05$ between the marked columns.

and 40% RH are presented in Fig. 1 in comparison with static cultures. The results are expressed as the percentage of normalized mucin secretion from the stressed cultures out of normalized secretion from static cultures (*white columns*). For each field of WSS tested, exposure of the cells to the WSS resulted in a significant increase in mucin secretion immediately after removal of the stimulus, with respect to the static cultures (Fig. 1 *a*). In addition, a significant difference in secretion was found between cultures exposed to WSS field with $\tau_{max} = 0.5$ and 5.0 dyne/cm^2. No significant differences were found in the secretion measured 24 h after the termination of the experiment in stressed cultures with respect to static cultures (Fig. 1 *b*).

Mucin secretion in response to oscillatory WSS at various climate conditions

Mucin secretion in response to WSS under different climate conditions was studied for the following environments: 1), 25°C and 80% RH; 2), 40°C and 40% RH; and 3), 40°C and 80% RH. In these experiments, the cell cultures were exposed to a WSS field with $\tau_{max} = 0.5$ dyne/cm^2 and $T_p = 4$ s for 15 min. For comparison, cell cultures were kept under static conditions for 15 min at similar temperature and humidity levels. Mucin secretion measurements were performed immediately and 24 h after the termination of the experiment. Two different data analyses were performed.

First, we performed an analysis to test whether different climate conditions affect the secretion of mucins from static cultures and from cultures under WSS. The graphs in Fig. 2 show the percentage of normalized mucin secretion from cultures that were exposed to different climate conditions out of normalized secretion from cultures at 25°C and 40% RH (*white columns*). Mucin secretion results obtained immediately and 24 h after exposure of static cultures to different climate conditions for 15 min are presented in Fig. 2, *a* and *c*. Mucin secretion results obtained immediately after exposure to WSS at different climate conditions and after 24 h are depicted in Fig. 2, *b* and *d*.

Mucin secretion from static cultures at 25°C/80% RH and 40°C/80% RH was significantly lower (i.e., ~30%) than that obtained from static cultures at 25°C/40% RH (Fig. 2 *a*). Insignificant differences were found between static cultures at 40°C/40% RH and 25°C/40% RH. On the other hand, mucin secretion from cultures exposed to WSS at 40°C/40% RH was significantly higher (i.e., ~30%) than secretion from cultures exposed to WSS at 25°C/40% RH (Fig. 2 *b*). Insignificant differences were found between cultures that were exposed to WSS at 25°C/80% RH and 40°C/80% RH and cultures that were exposed to WSS at 25°C/40% RH. Mucin secretion measured 24 h after the experiment was not significantly different in cultures that were exposed

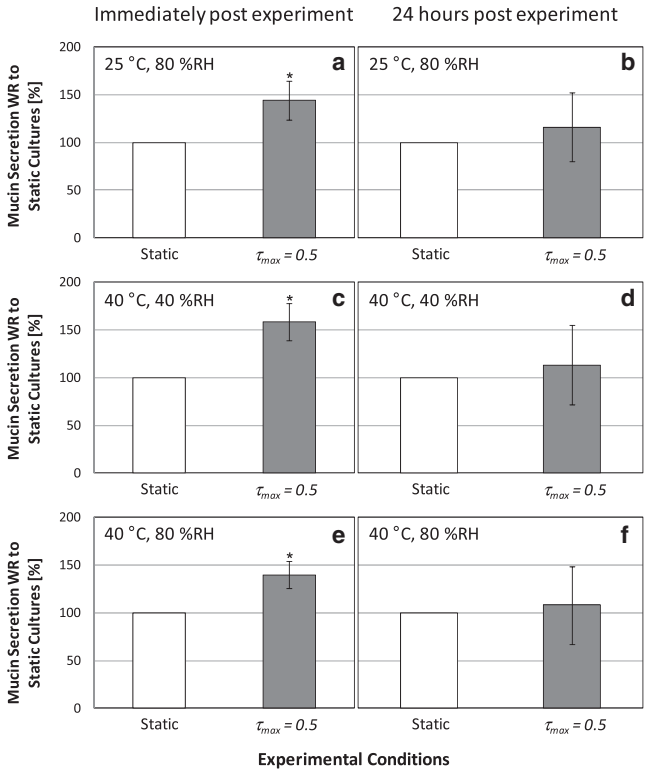

FIGURE 3 Mucin secretion measured (*a*, *c*, and *e*) immediately and (*b*, *d*, and *f*) 24 h after exposure of NEC cultures for 15 min to oscillatory WSS with $\tau_{max} = 0.5$ dyne/cm^2 and $T_p = 4$ s at different climate conditions in comparison with static cultures under the same climate conditions. The results are expressed as the mean ± SD of the percentage of normalized mucin secretion from cultures that were exposed to WSS under different sets of climate conditions with respect (WR) to normalized secretion from static cultures under the same conditions (*white columns*). *$p <$ 0.05, with respect to static cultures under the same climate conditions (*white columns*).

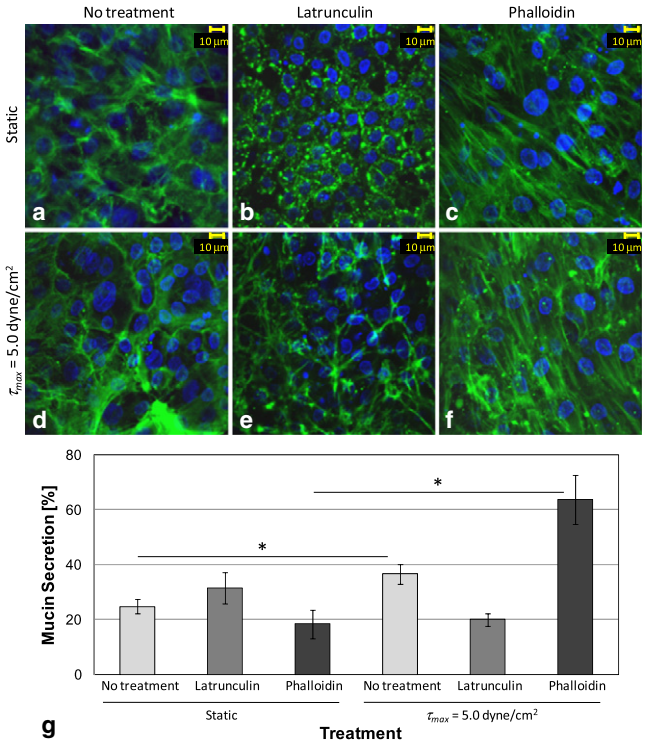

FIGURE 4 Representative images of actin MFs in (*a* and *d*) untreated, (*b* and *e*) Latrunculin-treated, and (*c* and *f*) Phalloidin-treated NEC (*a–c*) static cultures and (*d–f*) cultures that were exposed to oscillatory WSS with $\tau_{max} = 5.0$ dyne/cm^2 and $T_p = 4$ s, for 15 min at 25°C and 40% RH. Bar = 10 μm. (*g*) Mucin secretion from untreated, Latrunculin-treated, and Phalloidin-treated NEC cultures exposed to the same WSS conditions in comparison with static cultures. The results are expressed as the mean ± SD of normalized mucin secretion. *$p < 0.05$ between the marked columns.

to different climate conditions under WSS, or in static cultures (Fig. 2, *c* and *d*).

A different analysis of mucin secretion after exposure to WSS under different climate conditions is shown in Fig. 3, in which secretions obtained from static cultures under the same climate conditions are compared (*white columns*). Mucin secretion immediately after exposure to WSS under each condition significantly increased (by ~50%) compared with static cultures at the same climate conditions (Fig. 3, *a*, *c*, and *e*). Insignificant differences in mucin secretion were found 24 h after the termination of the experiment between cultures under WSS and static cultures at the same climates (Fig. 3, *b*, *d*, and *f*). This suggests that the effect of WSS on mucin secretion is independent of climate conditions, at least for the conditions tested.

Role of cytoskeletal components in mucin secretion under WSS

We tested the hypothesis that changes in mucin secretion under WSS are related to dynamic modifications of actin MFs and MTs by treating the NECs with disrupting and stabilizing agents before the application of WSS. Treatment of the NEC cultures with Latrunculin interfered with the polymerization of actin MFs, as can be seen in Fig. 4, *b* and *e*. The disassembly of the actin MFs by Latrunculin into short bundles and small aggregations of actin monomers was very obvious in the static cultures (Fig. 4, *b* versus *a*), whereas in the cultures under WSS it was less prominent (Fig. 4, *e* versus *d*). The effect of Phalloidin on the actin MFs (Fig. 4, *c* and *f*) was also clear in comparison with untreated cultures (Fig. 4, *a* and *d*). Phalloidin stabilized the actin MFs, resulting in many filamentous structures in the cultures as well as many stress fibers in both the static cultures and the cultures exposed to WSS in comparison with the untreated cultures. However, in the Phalloidin-treated cultures that were exposed to WSS (Fig. 4 *f*), more actin was observed in comparison with the Phalloidin-treated static cultures (Fig. 4 *c*).

Mucin secretion results obtained from Latrunculin- or Phalloidin-treated and untreated cultures that were exposed to oscillatory WSS in comparison with treated and untreated static cultures are shown in Fig. 4 *g*. A significant increase in mucin secretion was found in the Phalloidin-treated cultures

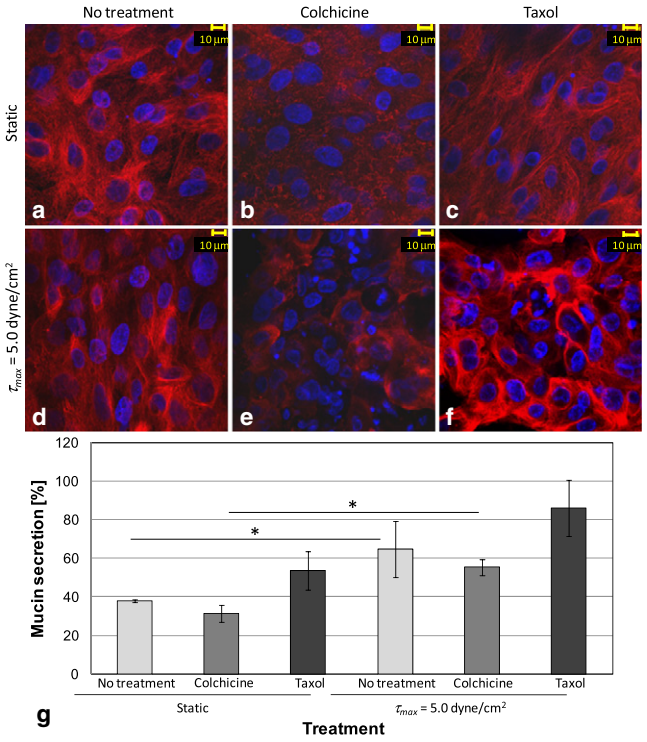

FIGURE 5 Representative images of β-tubulin in (*a* and *d*) untreated, (*b* and *e*) Colchicine-treated, and (*c* and *f*) Taxol-treated NEC (*a–c*) static cultures and (*d–f*) cultures that were exposed to oscillatory WSS with $\tau_{max} = 5.0$ dyne/cm^2 and $T_p = 4$ s, for 15 min at 25°C and 40% RH. Bar = 10 μm. (*g*) Mucin secretion from untreated, Colchicine-treated, and Taxol-treated NEC cultures exposed to the same WSS conditions in comparison with static cultures. The results are expressed as the mean ± SD of normalized mucin secretion. *$p < 0.05$ between the marked columns.

that were exposed to WSS in comparison with Phalloidin-treated static cultures. In fact, the increase in secretion in response to WSS was much higher in the Phalloidin-treated cultures (~240%) than in the untreated cultures (~50%). This suggests that actin-MF polymerization contributes to mucin secretion in response to WSS. Mucin secretion from the Latrunculin-treated cultures under WSS seemed to decrease in comparison with the secretion from the Latrunculin-treated static cultures; however, there was no statistically significant difference between the two. The fact that the secretion in the Latrunculin-treated culture under WSS was not significantly different from that of the Latrunculin-treated static culture strengthens the finding that actin-MF assembly encourages mucin secretion in response to WSS, since the disassembly of actin MFs by Latrunculin inhibited the expected increase in mucin secretion due to WSS.

Treatment of the NEC cultures with Colchicine interfered with the polymerization of MTs (Fig. 5, *b* and *e*) in comparison with untreated cultures (Fig. 5, *a* and *d*). Whereas the MTs in the Colchicine-treated static cultures showed broken filaments breaking into short fragments (Fig. 5 *b*), they had a blurry appearance in the Colchicine-treated cultures under WSS, probably because they were disassembling into very small subunits or even monomers (Fig. 5 *e*). The effect of Taxol on the assembly of MTs (Fig. 5, *c* and *f*) was also clear in comparison with untreated cultures (Fig. 5, *a* and *d*). Taxol stabilized the MTs, resulting in very strong filamentous structures in the static cultures and an even stronger stain of MTs in the cultures under WSS in comparison with the untreated cultures.

Mucin secretion results obtained from Colchicine- and Taxol-treated and untreated cultures that were exposed to WSS in comparison with treated and untreated static cultures are shown in Fig. 5 *g*. A significant increase in mucin secretion was found in the Colchicine-treated cultures that were exposed to WSS in comparison with Colchicine-treated static cultures. The increase in mucin secretion in response to WSS in Colchicine-treated cultures and in untreated cultures was similar (~70%). This suggests that the disassembly of MTs did not affect mucin secretion in response to WSS. Mucin secretion from the Taxol-treated cultures under WSS seemed to increase in comparison with the secretion from Taxol-treated static cultures; however, there was no statistically significant difference between the two. To assess the involvement of the MTs in mucin secretion under WSS, we used additional data from a similar study, which used a steady WSS of 1.0 dyne/cm^2 (15). Mucin secretion results from that study showed that the secretion from Taxol-treated cultures under WSS significantly increased in comparison to the secretion from Taxol-treated static cultures and by almost the same amount as in untreated cultures. Together, these results suggest that the MT assembly and disassembly dynamics is not directly related to mucin secretion in response to WSS.

DISCUSSION

Mucin secretion in response to WSS

Mucin secretion from airway goblet cells has never been systematically measured in response to WSS. Tarran et al. (29) applied phasic rotational WSS on cultures of normal bronchial epithelial cells and suggested indirectly that WSS induces mucin secretion. The magnitudes of physiological WSS tested in that study (0.06–6 dyne/cm^2) were similar to these used in the study presented here. It is presumed that WSS induces mucin secretion via ATP-induced purinergic signaling (11,30). Tarran et al. (29) showed that WSS induces the release of ATP from the cells so that the ATP concentration in the airway surface liquid is high enough to induce Cl^- secretion via P2Y2 receptor-mediated ion channel activation. The activation of P2Y2 receptors by ATP also results in elevation of intracellular Ca^{2+} (31), which has been shown to modulate mucin secretion (32,33).

ATP release in response to WSS was shown to induce fluid secretion in the airways, hydrating the periciliary liquid and the mucus layer (32). Moreover, it seems that physiological

oscillatory WSSs are required for airway surface liquid regulation (29). It is possible that the secretion of water in response to mechanical stimulation reduces mucin concentration in the airway surface liquid, which may be the driving force for secretion of mucins in response to the same stimulation to facilitate a return to the normal concentration of mucins on the surface. Whereas fluid secretion was shown to be induced by cyclic compression forces and not by steady forces (34), a previous study from our laboratory showed that mucin secretion is also increased in response to steady WSS (15). This requires further investigation; however, several hypotheses can be proposed regarding the mechanism by which WSS induces mucin secretion.

The Bernoulli principle states that the increase in speed of a fluid along a streamline occurs simultaneously with a decrease in the pressure and the potential energy. When a fluid enters into a constriction, its speed increases to satisfy mass conservation, and as a result its pressure is decreased. When air is driven from the environment into the nose, and then from the nostrils into the turbinates region (or, in the experimental system, from the air source into the flow chamber), the cross-sectional area for flow is remarkably decreased, resulting in increased airflow velocity in the nasal passages to satisfy mass conservation. As a result, the pressure near the surface epithelial cells must decrease due to conservation of energy (i.e., the Bernoulli principle). The resultant pressure gradient between the intracellular compartment and the epithelial surface may lead to mucin secretion from surface goblet cells onto the epithelial surface. This presumed pressure gradient may induce not only mucin secretion but also secretion of other substances, such as water, which is known to be coupled with secretion of mucins (32). It should be noted that although the nasal passages are not horizontal, air density is negligible and thus differences in potential energy are very small. In addition, it is possible that the drop in the surface pressure increases the pressure difference between the nasal tissue underneath the epithelium and the airway lumen, which may induce mucin secretion under WSS. In future studies, investigators could test this potential explanation by decreasing the surface pressure using a technique other than application of WSS (for example, by applying a vacuum on the culture surface).

WSS-induced secretion of mucins is achieved without any permanent damage to the cell membrane (15), and it is well established that this process is regulated by extracellular ligands. Hence, it is likely that WSS affects mucin secretion via a controlled signaling pathway, probably through ATP-induced purinergic signaling. However, the exact mechanism is currently unknown. Because ATP is assumed to be released either via ATP-permeable channels or by rapid exocytosis of ATP-containing granules (11,32), it is possible that WSSs mechanically change the conformation of transmembrane proteins that constitute an ATP-permeable channel. Stretch-activated ion channels have been shown to exist (8,9), and shear stress-activated channels have been identified in endothelial cells (35). On the other hand, it is possible that ATP is released via a more complex pathway in which another cellular structure (e.g., a specific integrin, primary cilia, or another receptor) is influenced by the WSS or by the resultant pressure gradient, and then a cascade of signaling occurs that eventually results in ATP release.

Mucin secretion in response to different climate conditions

In static cultures, mucin secretion significantly decreased at climate conditions of 25°C/80% RH and 40°C/80% RH with respect to cultures at 25°C/40% RH (Fig. 2 *a*). On the other hand, in the cultures exposed to WSS at a climate of 40°C/40% RH, the secretion significantly increased with respect to that at 25°C/40% RH (Fig. 2 *b*). These results may be explained by the coupling between mucin secretion and airway hydration (32,34). If water and mucin secretions are driven by the same factors, then in static conditions, when there is moisture in the cell environment, the cell would reduce the amounts of water and mucins secreted onto the cell surface. Under WSS, the culture surface is probably more dehydrated. In this case, when there is a relatively high humidity level in the cell environment (i.e., 80% RH) or when the temperature is relatively comfortable (i.e., 25°C), there is no need to increase water secretion and the accompanying mucin secretion. However, when WSS is applied at high temperature (i.e., 40°C) with no addition of moisture (i.e., 40% RH), the surface may be dehydrated to such an extent that mucin secretion is increased together with fluid secretion.

The changes in water content on the surface can be related to mucin concentration in the surface liquid. Because the epithelium responds to changes in the surface water content by absorbing or secreting water (32), it is possible that high humidity (i.e., 80% RH) at static conditions results in absorption of liquids. This would increase mucin concentration on the surface, and thus fewer mucins would be secreted in comparison with moderate humidity conditions (i.e., 40% RH). Likewise, high temperature (i.e., 40°C) with moderate humidity, at flow conditions, would induce water secretion, reducing the mucin concentration on the surface and leading to mucin secretion in comparison with comfortable temperature conditions. To conclude, it is possible that the combined effect of WSS, temperature, and humidity conditions influences mucin secretion, probably via regulation of surface water content, which dictates changes in mucin concentration on the surface.

Potential role of the cytoskeleton in mucin secretion under WSS

Mucin secretion in vitro has been shown to occur within tens of milliseconds in explants of canine trachea (21,36), rat

nasal mucosa (37), and human nasal and tracheobronchial mucosa (33). If the cytoskeleton is involved in this process, it should rearrange in the same timescale. In this study, we found that polymerization of actin MFs increased mucin secretion under WSS, whereas the dynamics of MT polymerization showed no effect (Figs. 4 *g* and 5 *g*). The role of the cytoskeleton, and especially that of actin MFs, in mucin secretion is controversial, and these results provide more evidence to be considered in the discussion.

Treating NECs with Phalloidin and exposing them to WSS resulted in increased mucin secretion (Fig. 4 *g*). Treatment with Latrunculin resulted in insignificant differences between secretions from static cultures and cultures exposed to WSS. Of note, enforcing polymerization of actin with Phalloidin increased the secretion only in the presence of WSS, and this increase was significantly higher than that in nontreated cultures. Treating the cells with Latrunculin prevented the increase in mucin secretion that was expected under WSS. Together, these results suggest that actin polymerization is involved in mucin secretion resulting from WSS. In addition, WSS has been shown to induce actin MFs polymerization in other types of cells (38,39). Thus, if WSS increases mucin secretion through an increase in actin MF polymerization, it can be argued that treating the cells under WSS with Phalloidin creates a synergistic effect of both actin-polymerization inducers, which could explain the very high increase in mucin secretion in those cells.

The cortical actin separates the cytoplasmic organelles from the plasma membrane, and thus has a role in regulating vesicle trafficking across the plasma membrane. However, although cortical actin was argued to function as a barrier that can mediate the transport of mucin granules toward the apical plasma membrane for secretion (40), its polymerization was observed during exocytosis in PC12 rat adrenal medulla cells, where Cdc42 was shown to trigger actin MF polymerization and to be essential for secretion (41). The results presented here are consistent with the latter.

Actin polymerization may contribute to mucin secretion by one or more of the following ways: 1), it can align the mucin granules with respect to docking sites of the plasma membrane and regulate their fusion with the plasma membrane (42); 2), it can physically push out the content of the granules upon fusion (43); and 3), it can stabilize the fused granules while secreting their content (44). Additionally, it has been established that upon agonist stimulation, myristoylated alanine-rich C kinase substrate (MARCKS) transiently localizes to the mucin granules and also interacts with actin and myosin, linking the granules to the cellular contractile machinery for movement to the cell periphery and subsequent release (10,45). This could potentially be one more way in which actin contributes to mucin secretion; however, although the role of MARCKS in the regulation of cortical actin cytoskeleton is well established, its role in exocytosis is controversial (46,47).

The MTs serve as transport tracks along which mucin secretory granules move from the Golgi to the cell apical region before secretion (48). Treating the cells with Colchicine and exposing them to WSS resulted in a significant increase in mucin secretion with respect Colchicine-treated static cultures, by approximately the same amount as in untreated cultures (Fig. 5 *g*). Treating the cells with Taxol seemed to increase mucin secretion in response to WSS in comparison with similarly treated static cultures, but the difference was statistically insignificant. Yet, exposing the Taxol-treated cells to steady WSS (15) resulted in a significant increase (in comparison with static cultures) that was almost the same as the increase observed in untreated cultures. These results suggest that MT polymerization and depolymerization do not affect mucin secretion in response to WSS.

This finding is not consistent with the known role of MTs in transporting mucin granules from the Golgi to the apical plasma membrane (48). However, these results are in good agreement with results from a study with rabbit intestinal goblet cells (49). The authors suggested that although MTs facilitate granule translocation by providing directed tracks for granule movement, their dynamics is not the motile mechanism that transports mucin granules to the apical plasma membrane for secretion. It could also be suggested that there are enough mucin granules that are already near or even docked to the plasma membrane waiting to be secreted upon stimulus, or that have already been transmitted from the MTs to the peripheral actin cytoskeleton, as is known to occur during the secretion process (10). Together with our results, these findings suggest that under WSS, actin (and not MTs) is the dynamic cellular transporter of mucin granules.

Self-recovery of NECs from mechanophysical stimulations

In this study, all changes in mucin secretion in response to the mechanophysical stimulations appeared immediately upon removal of the stimulus. A clear returning-to-normal behavior was demonstrated 24 h after removal of the stimulus (Figs. 1 *b*; 2, *c* and *d*; and 3, *b*, *d*, and *f*), suggesting that the effects of these stimulations are temporary. This outcome implies that no permanent damage was done to the cells by the levels of stimulations tested in this study. This conclusion is strengthened by the finding that the number of cells did not differ between static cultures and cultures exposed to steady WSS either immediately or 24 h after the experiments (data not shown). This means that the cells remained attached to the substrate and did not detach due to the stress. Furthermore, the confluency level of the static cultures and cultures exposed to WSS was always high, which also indicates that the epithelial structure was not damaged by the flow.

Cellular responses to oscillatory versus steady WSS

In this study, mucin secretion was increased in response to oscillatory airflow-induced WSS. In a previous study, we demonstrated that mucin secretion was also increased in response to steady flow. As the two flows differ in nature, it is impossible to compare these increases. Other studies also observed different responses to steady and nonsteady stresses. For example, ATP release from human tracheobronchial epithelial cells was increased in response to cyclic compression forces but was unaffected by nonoscillatory stresses (34). Bovine aortic endothelial cells exposed to pulsatile WSS with a positive mean value demonstrated an actin distribution similar to that of cells exposed to steady WSS (50). However, when the cells were exposed to pure oscillatory WSS, no orientation of the actin was observed and its distribution was diffuse (50). Other cellular responses in endothelial cells, such as increased intracellular free calcium or acidification of a perfusing buffer, were also inconsistent under steady and oscillatory flows (51,52). These data suggest that oscillatory flows, which are closer in nature to physiological flows, have a role in regulating biological processes in our body.

Nasal sensation and clinical aspects of mechanophysical stimulations

In this study, we observed clear responses of NECs to mechanophysical stimulations; however, the exact mechanisms by which these stimulations are sensed by the cells remain to be explored. It was previously suggested that the nose is rich with mechano- and thermoreceptors, but very little is known about their structure, location, and function (53). Previous experiments suggested that receptors of the nasal trigeminal nerve sense flow, pressure, and temperature in cats and rats (54,55); however, in humans they were shown to play no part in airflow sensation (56). Integrins or primary cilia on the NECs surface may also function as mechanoreceptors. In fact, primary cilia have been shown to sense shear stress in endothelial cells (57) and to induce Ca^{2+} influx in response to flow in renal cells (58). Such an influx may induce mucin secretion (10,32,33) as well as numerous other cellular responses.

Our goal in this study was to mimic physiologically realistic environments of NECs rather than pathological conditions. Nevertheless, WSS may also influence pathological conditions. For example, in patients with septal deviation, WSS magnitudes in the deviated areas are changed because of the altered geometry, and therefore mucin secretion in these areas would be expected to vary. Another example is the formation of contact points between the mucosal tissues on the lateral walls and the nasal septum, which may be associated with different types of headaches that are present in the absence of inflammation or infection (59). These contact points are formed as a result of anatomical variations, especially septal spurs and abnormalities of the turbinates (60). In narrowed nasal passages, air velocity is increased and thus the magnitude of WSS on the epithelial surface is increased in these areas. It is possible that the increased WSS levels, which were shown here to induce mucin secretion, increase the secretion in these areas locally and hence initiate the contact between facing mucosal tissues.

CONCLUSIONS

The nasal epithelium is exposed to mechanophysical stresses as a result of its exposure to respiratory airflows. In this work we used a laboratory model of the nasal epithelium to study the effects of WSS on mucin secretion at different climate conditions. Mucin secretion increased in response to WSS with respect to static cultures, independently of the airflow temperature and humidity. In static cultures, the secretion was reduced at high humidity levels with or without elevation of the temperature with respect to comfortable climate conditions. In cultures exposed to WSS, the secretion increased at high temperature with respect to cultures at comfortable climate. These results suggest that stresses that tend to dehydrate the epithelial surface lead to secretion of mucins. The polymerization of actin MFs was shown to increase mucin secretion under WSS, whereas the dynamics of MT polymerization did not affect the secretion. This study provides for the first time (to our knowledge) comprehensive data on mucin secretion in NECs due to airflow-induced mechanophysical stimulations of WSS at different levels of temperature and humidity.

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