High-Mobility Group Box 1 Upregulates MUC5AC and MUC5B Expression in Primary Airway Epithelial Cells

To the Editor:

High-mobility group box 1 (HMGB1) is a multifunctional protein. It is a nonhistone chromatin-binding protein that regulates chromatin structure and transcription, and after cellular release, it functions as a damage-associated molecular pattern to activate and sustain inflammatory responses. HMGB1 is increased in the BAL and sputum of patients with chronic respiratory illnesses such as chronic obstructive pulmonary disease (1) and cystic fibrosis (CF) (2), and functions as a biomarker (2, 3) for lung disease severity in patients with CF. HMGB1 binds and activates Toll-like receptor 2 (TLR-2), TLR-4, and TLR-9 (4), and receptor for advanced glycation end products (RAGE) (5). In murine models of *Pseudomonas aeruginosa* pneumonia, airway HMGB1 levels are increased and inhibition of HMGB1 rescues impaired phagocytic function and improves mouse survival (6). Intratracheal administration of HMGB1 upregulates IL-1β, TNF-α, and macrophage inflammatory protein 2, resulting in increased lung neutrophilic inflammation (7). Thus, HMGB1 is not only a biomarker but also a mediator of airway neutrophilic inflammation.

Previously, we reported that neutrophil elastase (NE), a major protease in the airways of patients with CF, induces the release of HMGB1 both *in vivo* and *in vitro* (8). NE also upregulates expression of MUC5AC (9), one of the major secreted airway mucins. Therefore, we hypothesized that an HMGB1 interaction with RAGE may have a previously unrecognized activity in the lung, i.e., upregulating the expression of two major airway mucins: MUC5AC and MUC5B. To test this hypothesis, we grew primary normal human bronchial epithelial (NHBE) cell cultures at the air–liquid interface and exposed them to recombinant HMGB1 in the presence or absence of a RAGE inhibitor. NHBE cells from at least two different deidentified donors were obtained from tracheal remnants after lung transplantation according to an institutional review board–approved protocol. After one passage for expansion, the cells were cultured in defined, serum-free media on Transwell inserts under submerged conditions until they reached confluency and then under air–liquid interface conditions for 9–11 days as previously described (10). The cells were stimulated with HMGB1 (10 or 100 ng/ml) or control vehicle in the apical and basolateral compartments for 24 or 48 hours. The apical media were collected and used in microtiter plate assays (11, 12) (5–50 μl) to determine the relative quantities of secreted MUC5AC protein (anti-MUC5AC monoclonal antibody: 45M1; GTX23659; GeneTex) and secreted MUC5B protein (anti-MUC5B rabbit polyclonal antibody: H-300; SCBT). The cells were lysed to harvest total RNA for qRT-PCR to quantitate *MUC5AC* mRNA, *MUC5B* mRNA, and *AGER* (RAGE gene) mRNA. Total cellular lysate protein (50 μg) was evaluated by Western blot analysis for RAGE expression (anti-RAGE rabbit polyclonal antibody: PA1-075; Thermo Fisher Scientific), normalized to β-actin, and band densities were expressed as a percentage of control treatment conditions. Statistical analyses for mRNA and relative protein expression were performed using ANOVA with *post hoc* comparisons. $P < 0.05$ was considered a statistically significant difference between treatment conditions.

In a separate set of experiments, RAGE was inhibited using FPS-ZMI (13) (553030; Millipore-EMD Biosciences), which was added to the apical and basolateral media 1 hour before stimulation with HMGB1 or vehicle control for 24 hours. Total cell RNA was isolated for qRT-PCR to evaluate the effect of RAGE inhibition on HMGB1-induced *MUC5AC* and *MUC5B* mRNA expression. For details regarding the methods used, please refer to the data supplement.

Here, we demonstrated that HMGB1 increased both *MUC5AC* and *MUC5B* mRNA and secreted protein in NHBE cells (Figures 1A–1D). The concentration of HMGB1 required to increase mucin expression is within the range detected in CF sputum (3). Inhibiting RAGE with a pharmacologic inhibitor was sufficient to block HMGB1-induced *MUC5AC* and *MUC5B* expression (Figures 1C–1D), supporting the idea that RAGE activation is necessary for mucin gene regulation. Interestingly, HMGB1 upregulated *AGER* mRNA (RAGE gene) and protein levels in NHBE cells (Figures 1E–1G), consistent with a mechanism of sustained HMGB1-RAGE activation in the airway. Given the strong association between airway HMGB1 and the risk for pulmonary exacerbations and lung disease progression in patients with CF, we speculate that HMGB1-induced mucin production and secretion may contribute to increased airway mucin concentrations during CF pulmonary exacerbations.

Our results are contrary to previous observations made by Kim and colleagues using the lung cancer cell line NCI-H292 (14). The authors reported that HMGB1 upregulated *MUC8*; slightly upregulated *MUC2*, *MUC5AC*, *MUC6*, and *MUC7*; and did not regulate *MUC5B* expression. In contrast to their study, we used primary airway epithelial cells from at least two different donors instead of a lung cancer cell line, and incubated the cells for 24–48 hours, versus 12 hours in the previous study. One limitation of our report is that we have yet to examine the signaling mechanisms or kinetics of exposure required for upregulation of *MUC5AC* and *MUC5B* expression by HMGB1. HMGB1 upregulates *MUC8* mRNA via increased phosphorylation of ERK and JNK (14). In NHBE cells, HMGB1 promotes wound healing via MAPK and Smad-2 signaling (15). These findings support the notion that MAPK and TGF-β signaling intermediates are triggered by an interaction between HMGB1 and RAGE/TLR4. We predict that activation of NF-κB by HMGB1 ligation of RAGE may play a role in *MUC5AC* and *MUC5B* upregulation (16). The time dependence of HMGB1 regulation of mucin gene expression also requires further examination, as a delayed effect on mRNA upregulation suggests the requirement of an HMGB1-regulated secondary signal to activate mucin gene expression.

In summary, this report highlights a novel function for HMGB1 in the airways: upregulation of *MUC5AC* and *MUC5B* mRNA expression and protein secretion. Because NE stimulates the release

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This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

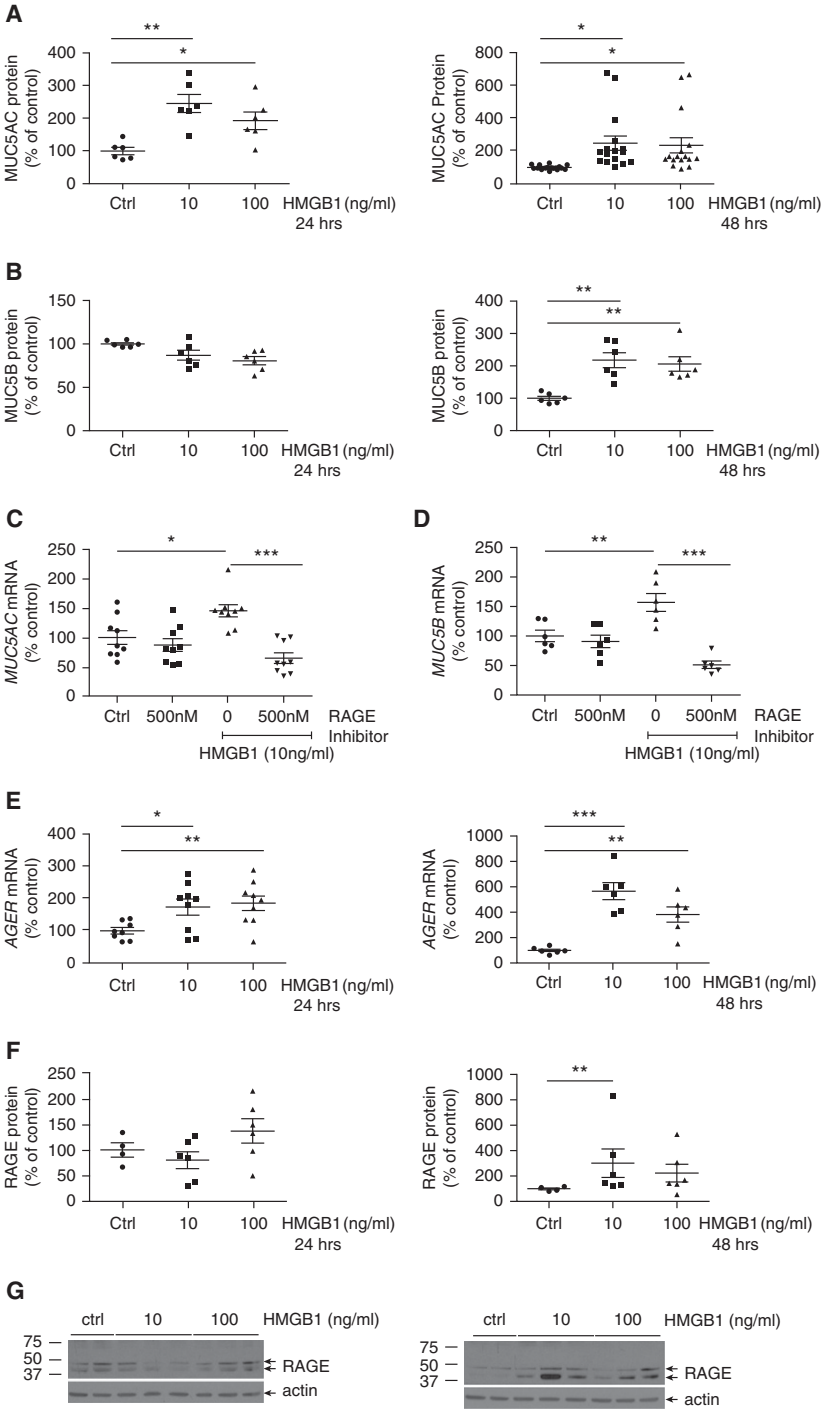

Figure 1. High-mobility group box1 (HMGB1) upregulated MUC5AC and MUC5B protein expression, and gene regulation was blocked by a receptor for advanced glycation end products (RAGE) inhibitor. Normal human bronchial epithelial (NHBE) cells were cultured at the air–liquid interface, treated with HMGB1 (10 or 100 ng/ml) for 24 or 48 hours, and analyzed for relative MUC5AC (*A*) and MUC5B (*B*) protein levels in the apical media by microtiter plate assay (*see* the data supplement). Involvement of the RAGE receptor in HMGB1-induced *MUC5AC* (*C*) and *MUC5B* (*D*) mRNA expression was tested by pretreatment with a RAGE inhibitor, FPS-ZMI (500 nM), for 1 hour, followed by administration of HMGB1 (10 ng/ml, 24 h). Expression of *MUC5AC* and *MUC5B* mRNA was determined by qRT-PCR as described in the data supplement. HMGB1 (10 or 100 ng/ml; 24–48 h) increased *AGER* (RAGE) mRNA expression (*E*) and RAGE protein levels as shown by densitometric analysis (*F*) of Western blots in well-differentiated, primary NHBE cells. A representative Western blot for RAGE is shown in *G*. *RAGE* mRNA was determined by qRT-PCR, and RAGE protein levels were compared with vehicle control–treated cells by Western blot, as described in the data supplement. Data are expressed as mean ± SEM. $P < 0.05$ was considered statistically significant (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ comparisons between conditions are marked by lines). Two to five independent experiments with six or more replicates per experimental treatment condition were performed using NHBE cells obtained from two to five different donors. Ctrl = control.

of HMGB1 into murine airways or culture media (8), it is possible that HMGB1 is required for NE-induced *MUC5AC* expression. Alternatively, HMGB1 ligation of RAGE may be an additional, nonredundant mechanism for upregulating airway mucin expression in chronic lung diseases such as chronic obstructive pulmonary disease and CF. ■

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β_2-Agonists Enhance Asthma-Relevant Inflammatory Mediators in Human Airway Epithelial Cells

To the Editor:

Inhaled β_2-agonists are frequently used therapies in asthma. They mediate their protective bronchodilator actions by inducing intracellular cAMP in smooth muscle cells. Although β_2-agonists have undoubted beneficial effects, safety concerns have been repeatedly raised regarding their use in asthma. Regular short-acting β_2 agonists (SABA) used in stable asthma result in worse asthma control than as-required use (1, 2), suggesting that use when not needed to relax contracted smooth muscle is actually harmful. Overuse of SABAs in the absence of inhaled corticosteroid (ICS) in asthma exacerbations has been repeatedly associated with increased risk of hospitalization or mortality (3). Long-acting β_2 agonists (LABA) use (likely in the absence of ICS) has also been linked to increased asthma mortality (4), although recent studies emphatically confirm their use *with* ICS is safe and beneficial (5, 6). A recent National Review of Asthma Deaths identified that 40% of deaths were associated with the prescription of too many SABA inhalers, and, in five deaths, LABAs were prescribed without ICS (7). The mechanisms underpinning these safety concerns have not been elucidated.

We have previously reported that IL-6, a proinflammatory cytokine (8, 9), is induced in bronchial epithelial cells (BECs) by salmeterol, but that induction disappeared when coadministered with ICS (8). Importantly, in relation to overuse of β_2-agonists in the context of asthma exacerbations, IL-6 induction by rhinovirus (RV) was further augmented by β_2-agonists (8). Promoter studies revealed that β_2-agonist augmentation of RV-induced IL-6 occurred via a cAMP response element (CRE) in the IL-6 promoter and, thus, the adverse effect (induction of IL-6 in BECs) occurs via exactly the same mechanism (induction of cAMP), as beneficial effects in smooth muscle cells (8). Another disease-relevant CRE-regulated gene, brain-derived neurotropic factor (BDNF), has

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