Airborne particulate matter increases MUC5AC expression by downregulating Claudin-1 expression in human airway cells

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CLB₂₀, a constituent of PM, induces secretion of multiple cytokines and chemokines that regulate airway inflammation. Specifically, IL-6 upregulates CLB₂₀-induced MUC5AC and MUC1 expression. Interestingly, of the tight junction proteins examined, claudin-1 expression was inhibited by CLB₂₀. While the overexpression of claudin-1 decreased CLB₂₀-induced MUC5AC expression, it increased the expression of the anti-inflammatory mucin, MUC1. CLB₂₀-induced IL-6 secretion was mediated by ROS. The ROS scavenger N-acetyl-cysteine inhibited CLB₂₀-induced IL-6 secretion, thereby decreasing the CLB₂₀-induced MUC5AC expression, whereas CLB₂₀-induced MUC1 expression increased. CLB₂₀ activated the ERK1/2 MAPK via a ROS-dependent pathway. ERK1/2 downregulated the claudin-1 and MUC1 expressions, whereas it dramatically increased CLB₂₀-induced MUC5AC expression. These findings suggest that CLB₂₀-induced ERK1/2 activation acts as a switch for regulating inflammatory conditions though a ROS-dependent pathway. Our data also suggest that secreted IL-6 regulates CLB₂₀-induced MUC5AC and MUC1 expression via ROS-mediated downregulation of claudin-1 expression to maintain mucus homeostasis in the airway. [BMB Reports 2017; 50(10): 516-521]

INTRODUCTION

The respiratory track in the human body has been highly exposed to extra-stimuli, such as air pollutants, viruses, bacteria, and microbes (1). Industrial development has induced a number of respiratory diseases. Now, many reports suggested that airborne particulate matter (PM) as important environmental pollutants induced many different diseases (2). PM is composed of transition metals, ions (sulfate, nitrate), quinoid stable radicals of carbonaceous material, minerals, reactive gases, and materials of biologic origin (2). Toxicological studies have indicated that PM induces many mechanisms of harmful cellular effects, such as radical-generating activity, activation of proinflammatory factors, DNA oxidative damage, and cytotoxicity (2). Airway mucus has an important factor to maintain the mucociliary clearance in the trachea and bronchi and acts to protect the lower airways and alveoli from PM and pathogens (3). Mucin hyperproduction and hypersecretion are frequently detected in many respiratory diseases such as airway infection, rhinitis, sinusitis, and otitis media (4). Mucin hyperproduction leads to decreased mucociliary clearance, increasing the chance of secondary infection because of other diseases in the airway (5, 6). Identifying the molecular mechanism underlying PM-induced mucin hyperproduction will enhance our understanding of mucin hypersecretion during PM-induced inflammation and provide the negative regulatory mechanism to develop novel therapeutic anti-PM reagents.

Tight junction proteins are expressed on the apical membrane of epithelia and play the critical roles of the barrier function and cell polarity. They consist of three groups of proteins: transmembrane proteins (occludin, Claudin, and junctional adhesion molecules); peripheral membrane proteins [ZO (Zonula occludens)-1, ZO-2, ZO-3, MUPP-1], which have PDZ (PSD95-Dlg-ZO1) domains and bind to transmembrane proteins, and cytoplasmic proteins (cingulin, 7H6 antigen, etc.) (7). The Claudin proteins are a family of major membrane proteins (24 identified), fundamental to the construction of tight junctions (8). By controlling the transfer of small ions and nutrients between cells, they keep cell-cell communication and homeostasis (9). Additionally, the Claudin are important for the preservation of differentiation, proliferation, cellular polarity, and so on (10). Even though they expressed throughout the lateral intercellular junction and nearby the basal cells that anchor the columnar epithelium to the basal lamina, expres-
sion of Claudin-1 and -4 could activate increased localization to the apical tight junction region (11). So far, the relationship between Claudin, PM, and airway mucins has not been studied.

Exposure of airway epithelium to PM induces cytokine/chemokine gene expression and increases the production of proinflammatory cytokines (12, 13). PM induces the increase in vascular permeability via the ROS-mediated calcium leakage via activated transient receptor potential cation channel M2 and via ZO-1 degradation by activated calpain (14). Although PM increased airway mucin expression and secretion (15, 16), the precise relationship between Claudin-1 and PM-induced MUC5AC and MUC1 during the airway inflammation and the mechanisms by which cytokines affect PM-induced MUC5AC and MUC1 expression have not been identified.

In this study, the effect of Claudin-1 on PM-induced mucin expression was investigated, and the relationship between Claudin-1 and airway inflammation in human airway epithelial cells was also established.

RESULTS

CLB2.0 secretes IL-6 extracellularly in normal human bronchial epithelial (NHNE) cells

Although collected PM2.5 was utilized at the top of some building at Dong-A University (Busan, Korea), the composition of PM2.5 is very variable depending on weather, skill, and so on. Indeed, Carboxyl Latex Beads (CLB) 2 μm (# C37278; ThermoFisher Scientific) should be used to get the same results. In addition, because PM contains so many heavy metals, the effect of heavy metals cannot be ruled out. CLB comprises carboxyl charge-stabilized hydrophobic polystyrene microspheres, and CLB2.0 has several different size beads with size ranging from 0.02 to 2.0 μm (3).

To determine whether CLB2.0 treatment controls the secretion of cytokines extracellularly that may regulate the respiratory microenvironment and affect tight junction proteins (TJs), we performed the cytokine array with cell culture medium after the treatment of NHNE cells with CLB2.0 for 4 h in a dose-dependent manner (Fig. 1A). The secretion of IL-6 increased dramatically in the cells in a dose-dependent manner (Fig. 1A, upper panel). In addition, the extracellular secretion of IL-6 reached its maximum level after 4 h of treatment with CLB2.0 (Fig. 1A, lower panel). To examine whether CLB2.0 stimulation is essential for IL-6 secretion and overproduction, IL-6-specific Western blot analysis (Fig. 1B) and ELISA (Fig. 1C) were performed. CLB2.0 did not induce IL-6 secretion and overproduction in a time-dependent manner.

MUC5AC was known to the inflammatory mucin, but MUC1 was recognized as anti-inflammatory mucin (17-20). Interestingly, MUC5AC gene expression increased significantly by the cotreatment of CLB2.0 and IL-6 compared to CLB2.0 alone (Fig. 1D), but the MUC1 gene expression dramatically decreased by the cotreatment of CLB2.0 and IL-6 (Fig. 1E). Therefore, IL-6 secreted by the CLB2.0 can control CLB2.0-induced MUC5AC and MUC1 gene expression in the airway epithelial cells. Therefore, the cells activated by CLB2.0 could promote the secretion and overproduction of IL-6, which may trigger signal transduction pathways to regulate the inflammatory microenvironment by either an autocrine or paracrine manner in airway epithelial cells.

Overexpressed Claudin-1 decreases CLB2.0 and IL-6-induced MUC5AC gene expression, but increases MUC1 gene expression

When the same experiments (Fig. 1) were carried out using human lung mucoepidermoid carcinoma cell line, NCI-H292 cells, we got the same results as in the normal cells (Supplement Fig. 1). Unfortunately, because primary cells have technical limitations, NCI-H292 cells were used.

We hypothesized whether CLB2.0 could affect tight junction proteins to invade airborne PM across airway epithelium. First, TJs such as Claudin-1, ZO-1, and E-cadherin were investigated. Claudin-1 expression was inhibited by CLB2.0, and the cotreatment of CLB2.0 and IL-6 dramatically inhibited Claudin-1 expression, but not ZO-1 and E-cadherin expression (Fig. 2A). To investigate whether secreted IL-6 could affect Claudin-1 expression, specific siRNA-IL-6 was used. Both recombinant
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Fig. 2. Effect of Claudin-1 on CLB20-induced MUC5AC and MUC1 gene expression. (A) After the cells were treated with CLB20 (10 mg/ml) or CLB20 and IL-6 (30 ng/ml) for 24 h, Western blot analysis was performed with specific Claudin-1, ZO-1, and E-cadherin antibodies. Tubulin was used as the loading control. (B) Cells were transfected with either a construct driving the expression of wild-type IL-6 or siRNA specific for IL-6. Cells were then treated with CLB2 (10 mg/ml) for 4 h prior to the generation of total cell lysates, and then Western blot analysis for Claudin-1 was performed. (C) Cells were transfected with either a construct expressing of wild-type Claudin-1 or siRNA specific for Claudin-1, followed by treating with CLB2 (10 mg/ml) for 24 h prior to the generation of total cell lysates, and then qRT-PCR for MUC5AC and MUC1 mucin genes were performed. *P < 0.05 compared to control, **P < 0.05 compared to CLB20-treated cells, and ***P < 0.05 compared to CLB20/wild-type Claudin-1-treated cells.

IL-6 and CLB20 completely diminished Claudin-1 expression, whereas siRNA-IL-6 partially inhibited Claudin-1 expression, suggesting that both IL-6 and secreted CLB20 could completely decrease Claudin-1 expression (Fig. 2B). Next, in order to investigate whether decreased Claudin-1 expression might affect airway inflammation, qPCR for MUC5AC (inflammatory mucin) and MUC1 (anti-inflammatory mucin) in NCI-H292 cells was performed. Interestingly, ectopic overexpressed Claudin-1 dramatically inhibited MUC5AC gene expression, but siRNA-Claudin 1 increased the CLB20-induced MUC5AC gene expression. In addition, the overexpressed Claudin-1 increased CLB20-induced MUC1 gene expression, but siRNA-Claudin 1 decreased significantly MUC1 gene expression (Fig. 2C). These results suggest that CLB20 could decrease Claudin-1, and thus the diminished Claudin-1 could not play its own physiological functions during CLB20-induced airway inflammation.

ROS production by CLB20 is mediated for inhibiting Claudin-1 expression in the airway epithelial cells
The PM could induce cytokine and chemokine expression to alter the inflammatory microenvironment. However, little is known about an intracellular mechanism of PM in regulating inflammation in airway epithelial cells. Accordingly, to investigate whether CLB20-induced could control inflammatory cytokine gene expression in the airway, ROS production was investigated. Cells were treated with CLB20 in a time-dependent manner, and ROS production was measured using DCF. ROS production peaked at 5 min and then decreased at 10 min (Fig. 3A). In addition, ROS production was unaffected cell viability (Fig. 3B). Moreover, cotreatment of CLB20 and IL-6 increases the ROS production much more than CLB20 alone (Fig. 3C). Next, to check whether ROS might mediate Claudin-1, IL-6, and MUC5AC gene expression, NAC was used. Inhibition of ROS production increased Claudin-1 expression, but decreased IL-6 and MUC5AC gene expression. Interestingly, the cotreatment of CLB20 and NAC increased MUC1 gene expression. These results suggest that ROS production is not only critical for the inhibition of Claudin-1 and the activation of MUC5AC gene expression in response to CLB20, but also essential for CLB20-induced secretion extracellularly of IL-6.
CLB2.0-induced activation of ERK1/2 is mediated by ROS and plays as a switch molecule to regulate the inflammatory condition

To examine which molecule is involved in the downstream signaling protein of ROS production within the signaling pathway of Claudin-1 and mucins gene expression induced by CLB2.0, the phosphorylation of MAPks by phospho-specific antibodies was investigated. MAPks are widely distributed in mammalian cells and can be activated by ROS. The phosphorylation of ERK1/2 by CLB2.0 reached a maximum at 15 min and decreased at 30 min after the CLB2.0 treatment, but not p38 and JNK MAPks (Fig. 4A). To check whether ERK1/2 activation is related to the ROS production after the treatment of CLB2.0, NAC was used (Fig. 4B). NAC dramatically decreased CLB2.0-induced ERK1/2 activation, suggesting that ROS might be critical for CLB2.0-induced ERK1/2 activation. Interestingly, the treatment with wild-type ERK1/2 inhibited CLB2.0-induced Claudin-1 and MUC1 expression, but increased MUC5AC gene expression. However, siRNA-ERK1/2 significantly increased CLB2.0-induced Claudin-1 and MUC1 expression and dramatically decreased MUC5AC gene expression (Fig. 4C).

**DISCUSSION**

Inhalation of approximately 12,000 L of air a day passing through the airway epithelium results in the accumulation of up to 25 million particles an hour (21). The first defense line against inhaled harmful particles on damaging the airway epithelium is the mucus overproduction and hypersecretion. Airway mucus is a critical element of the mucociliary clearance system in the respiratory tract, and thus plays to defend the lower airways and alveoli from exposure/infection of PM and pathogens (3). However, there is no evidence that mucus hypersecretion and overproduction may affect PM-induced airway inflammation in the human respiratory track. MUC5AC has been considered inflammatory mucin because many inflammatory mediators, air pollutants, and pathogens increased MUC5AC overproduction and hypersecretion to progress inflamed microenvironment (22-24), whereas MUC1 has been considered anti-inflammatory mucin to maintain homeostasis (17-20). In this study, we suggest that CLB2.0 disrupted the balance between the inflammatory condition and homeostasis to make inflamed condition in the airway. Thus, it is critical to understand the regulatory mechanism by which negative regulatory proteins/molecules decreasing the MUC5AC overexpression and hypersecretion in the airway epithelium are cleared and stimulants (chemical compounds) increase the MUC1 overproduction to maintain (restore) homeostasis in the respiratory track.

Recently, Wang et al. reported that PM could disrupt endothelial cell barrier via TJ degradation, thus increases vascular permeability (14). The same group also reported that PM disrupted human lung endothelial barrier via ROS- and p38 MAPK-dependent pathway (25). Furthermore, Caraballo et al. reported PM altered alveolar epithelial barrier via a decrease in occludin (26). Consequently, PM should disrupt the barrier to invade into cells via inhibiting TJs. Accordingly, we hypothesized that overexpressed TJs protein in the human airway epithelium might prevent the weakness of the membrane permeability. According to the result of cytokine array, concentrated IL-6 secreted extracellularly at early time by CLB2.0 (Fig. 1C). Extracellular IL-6 could transfer inflammatory signaling to the nearby cells and cells secreting IL-6, up-take IL-6 again (autocrine manner). In fact, Gao et al. suggested the importance of autocrine IL-6 signaling in lung cancer (27). The cotreatment of CLB2.0 and IL-6 significantly disrupted Claudin-1 compared to CLB2.0 only (Fig. 2A). This result provides very important evidence for TJs, because of the following reasons 1) CLB2.0 could secrete several cytokines/chemokines to completely disrupt TJs, 2) IL-6 could transfer inflammatory signaling to the nearby cells and synergistically amplify CLB2.0 signaling, and 3) if impaired TJs were restored by genetic method (gene-delivery system; e.g., transfection or viral infection), restored TJs could prevent CLB2.0 invasion because of much rigid membrane.

Oxidative stress has provided the important mechanisms for
PM-induced pro-inflammatory responses in the airway (28). Both primary ROS generation by PM and PM components could generate ROS production, as well as PM-exposed cells formed ROS and reactive nitrogen species via a secondary pathway (29). Short-term exposure to PM2.5 causes lung inflammation and mucus hypersecretion in mice, in a study that implicated EGFR signaling pathway activation (16). Similarly, ROS produced by CLB2.0 was strongly related to CLB2.0 implicated EGFR signaling pathway activation (16). In addition, CLB2.0-induced ERK1/2 activity was mediated by ROS, and then plays a switch molecule to progress either inflamed condition or homeostasis. This is an important finding, because ERK1/2 is critical signaling protein to increase the MUC5AC gene expression. In our previous reports, negative regulatory proteins (SHP-2 and IL-1ra) attenuate ERK1/2 activation to abolish MUC5AC gene expression in inflamed cells (24). Thus, understating the effects of ERK1/2 and Claudin-1 function is expected to provide effective information of the working mechanism of CLB2.0 toxicity, as well as for the development of innovative therapeutic medication against destructive human health roles of air pollution inhalation.

In summary, the results of this study support a novel working hypothesis in which CLB2.0 induces the intracellular secretion of IL-6 in the airway epithelial cells, primarily through autocrine or paracrine manner. This activation results in a CLB2.0/IL-6/ROS/ERK1/2-dependent increase in the MUC5AC gene expression, which in turn activates the expression/secretion of inflammatory cytokines/chemokines (Fig. 4D). In contrast, overexpressed Claudin-1 may overcome CLB2.0-induced toxicity/airway inflammation because of increased MUC1 gene expression and dampens the inflammatory response to enhance anti-inflammation in the airway microenvironment. This study suggests an anti-inflammatory role of Claudin-1 during the airway inflammation and provides the molecular mechanisms of CLB2.0-mediated immune responses in air pollutants inhalation.

MATERIALS AND METHODS

Materials
Carboxyl latex beads (4% w/v, 2 µm) were purchased from Thermo Fisher (C37278). The ELISA kit and purified cytokine of IL-6 were purchased from R&D Systems. The ROS inhibitor was purchased from Sigma-Aldrich.

Cytokine assay
The cytokine assay was performed using a Human Cytokine Array Panel A kit (R&D Systems) according to the manufacturer’s instructions. Briefly, serum-starved cells were treated with CLB2 for 4 h. After the treatment, the supernatant was assayed according to the kit instructions.

RT-PCR
Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories; Hercules, CA) with iQ SYBR Green Supermix. Reactions were performed in a total volume of 20 µl - including 10 µl 2× SYBR Green PCR Master Mix, 300 nM of each primer, and 1 µl of the previously reverse-transcribed cDNA template.

Statistical analysis
Data are presented as the mean ± S.D. of at least three independent experiments. Where appropriate, statistical differences were assessed by the Wilcoxon Mann-Whitney test. P-values less than 0.05 were considered statistically significant.

ACKNOWLEDGEMENTS
This study was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korea government (NRF-2016R1D1A1B03932521 for K.S.S.)

CONFLICTS OF INTEREST
The authors have no conflicting interests.

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