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Aquaporin 5 expression inhibited by LPS via p38/JNK signaling pathways in SPC-A1 cells

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ABSTRACT

Proper H_2O to mucin ratio of airway mucus is important for mucociliary clearance. Recent studies suggest that decreased aquaporin 5 (AQP5) is correlated with increased staining of MUC5AC in submucosal glands of COPD patients. Lipopolysaccharide (LPS) is one of the major insults in airway mucin secretion in COPD. In this study, changes in both AQP5 and MUC5AC expression levels in SPC-A1, a human airway submucosal gland cell line, were quantified after exposure of the cells to LPS. AQP5 transcription and protein expression were decreased while MUC5AC expression was increased by LPS exposure in SPC-A1 cells. Further studies revealed that AQP5 expression was down-regulated via the p38/JNK signaling pathway, while MUC5AC was up-regulated through the EGFR-p38/JNK pathway. Therefore, p38 and JNK may become promising targets to preserve AQP5 expression and prevent MUC5AC over-expression to restore proper H_2O to mucin ratio of the airway mucus, which may be beneficial to the clinical management of COPD patients.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is one of the major causes of chronic morbidity and mortality globally. It is currently the fourth leading cause of population death and is projected to rank the fifth as a worldwide burden of disease by 2020 (Rabe et al., 2007).

COPD is pathologically characterized by goblet and submucosal gland cell hyperplasia as well as mucus hypersecretion (Vestbo et al., 1996). Airway mucus is mainly composed of water and ions, within which approximately 2% is mucins (Rogers, 2003; Livraghi and Randell, 2007). Of the 20 known human MUC genes, MUC1, MUC2, MUC4, MUC5AC, MUC5B, and MUC6 are expressed in airways (Casalino-Matsuda et al., 2006). Among those mucin genes, MUC5AC and MUC5B are the major gel-forming mucin genes. Study showed MUC5AC is highly inducible while MUC5B is expressed constitutively (Rogers, 2004). In the airway, MUC5AC was thought to be produced by goblet cells rather than submucosal gland cells. However, recent studies show that MUC5AC is present in secretions from the human tracheal glandular ducts, suggesting that submucosal gland cells are also the source of MUC5AC (Roger et al., 2001; Caramori et al., 2009). In addition, it has been shown that COPD is associated with increased MUC5AC expression in submucosal glands (Ma et al., 2005; Inoue et al., 2008).

Recent studies suggested association between mucin secretion and Aquaporin 5 (AQP5) expression. The AQPs were a family of small (30 kDa monomer) integral membrane proteins that function as selective water transporters (Verkman, 2007). Thirteen related AQPs have been discovered in mammals and at least four of them were present in the lung and airway: AQP1 is expressed in microvascular endothelia, AQP3 and AQP4 are expressed in airway epithelia, and AQP5 is localized at apical membrane of type I alveolar epithelial cells, acinar epithelial cells in submucosal glands and large airway epithelia (Kreda et al., 2001; Chen et al., 2006a,b). Deletion of AQP5 resulted in more concentrated protein and mucus secretion in the upper respiratory tract of mice (Song and Verkman, 2001), possibly due to impaired fluid transport without AQP5 expression as seen in salivary gland secretion (Ma et al., 2000).

In COPD patients, decreased AQP5 expression is associated with increased staining of MUC5AC in their submucosal glands (Wang et al., 2007). Both AQP5 down-regulation and MUC5AC up-regulation could potentially result in dehydrated and viscous mucus. The mechanism for AQP5 down-regulation and MUC5AC up-regulation is not clear. AQP5 down-regulation could be an independent event or could result in MUC5AC up-regulation. Our previous study showed AQP5 down-regulation by siRNA in SPC-1 cell line was associated with MUC5AC over-expression (Chen et al., 2006b). It has been shown that bacterial endotoxins lipopolysaccharides

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(LPSs) are associated with mucin overproduction in COPD patients (Wang et al., 2009). In this study, SPC-A1, a human airway submucosal gland cell line, was used to quantify modulations of AQP5 and MUC5AC after LPS exposure, and assess possible signaling pathways leading to alterations in AQP5 and MUC5AC expression.

2. Methods and materials

2.1. Cell culture

SPC-A1, a human lung adenocarcinoma cell line, was provided by Shanghai Cell and Biology Institute, Chinese Academy of Science (Shanghai, China). SPC-A1 cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), penicillin (100 U/ml), and streptomycin (100 U/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. Before experiments, confluent SPC-A1 cells were serum-starved for 24 h to maintain low basal levels of MUC5AC and AQP5 expression.

2.2. Treatment of cells with LPS and inhibitors

After 24 h of serum starvation, SPC-A1 cells were stimulated with LPS (Sigma, USA) at various time and concentrations. To assess the role of EGFR in LPS-induced AQP5 transcription, the cells were treated with EGFR tyrosine kinase inhibitor AG1478 (Calbiochem, USA) at 2 and 10 μM. To decide how AQP5 transcription was affected by intracellular MAPK activation, the cells were treated with either ERK1/2 inhibitor PD98059 (Cell Signaling Technology, USA) at 2 and 10 μM, p38 inhibitor ML3404 (Calbiochem, USA) at 2 and 10 μM or JNK inhibitor SP600125 (Calbiochem, USA) at 2 and 10 μM. For inhibitor studies, serum-starved cells were pretreated with the inhibitors for 30 min before exposure to stimuli, the cells were then cultured for 6 h with both LPS and the inhibitors.

2.3. Quantitative real-time PCR

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen) following the manufacturer's instruction and was quantified by spectrophotometer. cDNA was reverse transcribed with MMLV first-strand synthesis kit (BBI, USA). PCR reactions were preformed in 25 μl-reaction containing 1 μl cDNA, 1 mM of each forward and reverse primer, and 0.25× SyBr green Mix. β-Actin was used as the internal control to quantitate initial cellular transcripts. Primer sequences included: β-actin—sense: 5′CCTGTACGCCAACACAGTGC3′, antisense: 5′ATACTCCTGCTTGCTGATCC3′; AQP5—sense: 5′CTGTCCATTGGCCTGTCTGTC3′, antisense: 5′GGCTCATACGTGCCTTTGATG3′; MUC5AC—sense: 5′GAGGGCAACAACGTCATCTCC 3′, antisense: 5′TCT TGGTCAGCCACCTTCACA3′. Real-time PCR conditions for amplification of target genes were as follows: pre-denaturing at 94 °C for 5 min, 40 cycles of denaturing at 94 °C for 10 s, annealing for AQP5 at 60 °C (MUC5AC at 59 °C, β-actin at 57 °C) for 15 s, extension at 72 °C for 20 s, followed by the last extension at 72 °C for 10 min. Amplification data measured by fluorescence were collected in real-time and analyzed by Rotor-Gene 6.0.14 Software.

2.4. Immunofluorescence staining

SPC-A1 cells were seeded into six-well tissue culture plates, with glass cover slips placed on the bottom. Once the cells were 70–80% confluent, the cover slips were removed. Cells were washed with PBS, fixed in 4% paraformaldehyde for 20 min, and then incubated with anti-AQP5 primary antibody (1:100 Calbiochem, USA) in a humidity chamber at 37 °C for 1 h. Cells were washed three times in PBS and incubated with goat anti-rabbit IgG antibody conjugated to FITC at room temperature for 1 h. After being washed three times in PBS, cells were visualized using a Zeiss microscope (Carl Zeiss, Germany).

2.5. Western blot analysis

Western blots were performed as described in the manufacturer's instructions (Wang et al., 2009). Briefly, western blots were performed using whole cell extracts, separated on 8–10% SDS-PAGE gels and transferred to polyvinylidine difluoride membranes (Pall Life Sciences, Pensacola, FL). The membrane was blocked with a solution of TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat milk. After three washes in TBS-T, the membrane was incubated with anti-AQP5 primary antibody (1:1000 Calbiochem, USA). After another three washes in TBS-T, the membrane was incubated with 1:2000 dilution of the corresponding secondary antibody. The membrane was reacted with chemiluminescence reagent ECL (Amersham Biosciences) to visualize the blots.

2.6. MUC5AC ELISA

After incubation of SPC-A1 cells at different conditions, levels of MUC5AC protein in cell culture supernatants and in cell lysates were measured by ELISA as previously described (Takeyama et al., 1999). The amount of MUC5AC protein in each tested portion was normalized to total protein and expressed as fold changes over control.

2.7. Statistical analysis

Data were expressed as means ± SEM. Statistical differences among multiple groups were calculated by using one-way analysis of variance (ANOVA). If a certain ANOVA was statistically significant, Student–Newman–Keuls test was used in multiple comparisons. Statistically significant differences were accepted at $p < 0.05$.

3. Results

3.1. LPS exposure progressively reduced AQP5 mRNA and protein expression in SPC-A1 cells

The indirect immunofluorescence staining showed that AQP5 was located in the membrane of SPC-A1 cells (Fig. 1A). The cells were incubated in media supplemented with 0, 5, 10, 20, and 40 μg/ml LPS for 6 h in real-time PCR or for 24 h in western blotting. AQP5 mRNA and protein levels decreased significantly with 10, 20 or 40 μg/ml LPS treatment in a dose-dependent manner (Fig. 1B). AQP5 mRNA and protein decreased maximally with 20 μg/ml LPS, thus 20 μg/ml LPS was used for subsequent experiments. We have routinely checked cell viability by MTT and found no evidence that the dose of LPS used in this study was harmful to the cultures (data not shown here).

To analyze the time course of LPS-mediated inhibition on AQP5 expression, SPC-A1 cells were treated with LPS for various periods of time before total RNA and protein isolation. AQP5 mRNA decreased significantly to about 20% of the control level after 6 h LPS treatment. Similarly, AQP5 protein decreased dramatically to about 20% of the control level after 12, 24 or 48 h of LPS treatment (Fig. 1C). AQP5 protein did not return to baseline level after 48 h.

3.2. LPS exposure progressively increased MUC5AC mRNA and protein expression in SPC-A1 cells

To determine changes of MUC5AC with different LPS doses, the cells were treated with different concentrations of LPS, i.e.