P. aeruginosa Lipopolysaccharide-Induced MUC5AC and CLCA3 Expression Is Partly through Duox1 In Vitro and In Vivo

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Abstract

Background: We have previously found that reactive oxygen species (ROS) are involved in Pseudomonas aeruginosa lipopolysaccharide (PA-LPS) induced MUC5AC in airway epithelial cells. Dual oxidase1 (Duox1), a member of NADPH oxidase (Nox), is known to be responsible for ROS production in respiratory tract epithelial cells. Our aim was to clarify whether Duox1 was also involved in the PA-LPS-induced MUC5AC and calcium dependent chloride channel 3 (Clca3), another recognized marker of goblet cell hyperplasia and mucus hyper-production.

Methods: PA-LPS-induced Duox1 mRNA levels were examined in A549 cells, primary mouse tracheal epithelial cells (mTECs) and lung tissues of mice. Nox inhibitors diphenyleneiodonium chloride (DPI) and Duox1 siRNA were used to investigate whether Duox1 is involved in PA-LPS-induced MUC5AC and Clca3 expression both in vitro and in vivo.

Results: Duox1 is induced by PA-LPS in A549 cells, primary mTECs and lung tissues of mice. DPI significantly inhibited PA-LPS-induced up-regulation of Duox1, Muc5ac and Clca3 in primary mouse trachea epithelial cells and lung tissues of mice. Knockdown of Duox1 markedly inhibited PA-LPS-induced MUC5AC expression via a ROS-TGF-β cascade in A549 cells. Furthermore, DPI significantly inhibited PA-LPS-induced increases in inflammatory cells accumulated in mouse lungs.

Conclusions: We demonstrate for the first time that PA-LPS-induced MUC5AC and Clca3 expression is partly through Duox1, and provide supportive evidence for Duox1 as a potential target in treatments of mucin over-production diseases.

Introduction

Mucus hypersecretion is commonly observed in chronic inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis. Excessive production of mucus contributes to morbidity and mortality in these diseases by plugging the airways and causing recurrent infections [1,2]. MUC5AC mucin is the major component of airway mucus [3,4]. A number of in vitro and in vivo studies have been carried out to explore the signaling mechanisms underlying the regulation of MUC5AC expression induced by many different stimuli [5,6,7,8,9,10]. Pseudomonas aeruginosa (PA) infection is common in chronic inflammatory airway diseases [11], especially in cystic fibrosis. It has been previously shown that Pseudomonas aeruginosa lipopolysaccharide (PA-LPS) significantly up-regulates MUC5AC mucin expression in airway epithelial cells [12,13,14]. However, the underlying molecular mechanisms remain largely unknown.

Reactive oxygen species (ROS) have been found to play important role in cigarette smoke, neutrophil elastase and phorbol 12-myristate 13-acetate (PMA) induced MUC5AC mucin expression [15,16,17]. In addition, we have previously shown that ROS are involved in PA-LPS induced MUC5AC production [18]. Dual oxidases (Duox), members of the NADPH oxidase (Nox) family, originally identified and cloned from the epithelium of the thyroid gland [19,20,21], were initially found to be responsible for ROS production, which is involved in the anti-microbial activity of lactoperoxidase in respiratory tract epithelial (TBE) cells [22]. The two Duox isoforms Duox1 and Duox2 have high structural similarity. While they were differentially regulated by a variety of
proinflammatory factors, Duox1 is induced by Th2 cytokines IL-4 and IL-13, whereas Duox2 is induced by Th1 cytokines IFN-γ and poly(I:C) [23]. In addition, Duox1 and Duox2 have distinct functions in airway epithelium. Duox2 is mainly involved in inflammation, whereas Duox1 is mainly responsible for mucus production [17]. Nadel et al [17] have recently reported that Duox1 mediated neutrophil elastase- and PMA-induced MUC5AC mucin expression in airway epithelial cells. However, it is still unclear if Duox1 could also be involved in LPS-induced MUC5AC mucin expression. We hypothesized that Duox1 may also mediate PA-LPS-induced MUC5AC expression via controlling ROS production in airway. Based on our previous finding that the ROS-TGF-α signaling pathways mediate PA-LPS-induced MUC5AC expression in NCI-H292 cells [18], we explored whether Duox1 controls the production of ROS and TGF-α in airway epithelial cells. To further confirm the specific role of Duox1 in mucus regulation, we also investigated whether knockdown of Duox2 can influence MUC5AC expression in A549 cells. Furthermore, because we previously showed that ROS scavenger di-methylthiourea (DMTU) inhibited PA-LPS-induced MUC5AC expression in vitro, we sought to further investigate whether DMTU also plays a similar role in vivo.

Figure 1. PA-LPS induced Duox1 expression in A549 cells, primary mouse trachea epithelial cells and in mouse lungs. A: PA-LPS (5 μg/mL and 10 μg/mL) significantly up-regulated Duox1 mRNA expression in A549 cells; B: PA-LPS (2.5 μg/mL) significantly up-regulated Duox1 mRNA expression in primary mTECs; C: PA-LPS (100 μg) significantly up-regulated Duox1 mRNA expression in lung tissues of mice. #P<0.05 compared with control, *P<0.05 compared with control. doi:10.1371/journal.pone.0063945.g001

Figure 2. Suppression of PA-LPS-induced Duox1, Muc5ac and Clca3 expression by DPI in primary mouse trachea epithelial cells. A: Diphenylene iodonium (2.5 μM) significantly inhibited PA-LPS-induced Duox1 mRNA expression in primary mTECs. B: Diphenylene iodonium (2.5 μM) significantly inhibited PA-LPS-induced Muc5ac mRNA expression in primary mTECs. C: Diphenylene iodonium (2.5 μM) significantly inhibited PA-LPS-induced Clca3 mRNA expression in primary mTECs. #P<0.05 compared with control, *P<0.05 compared with PA-LPS. doi:10.1371/journal.pone.0063945.g002
Calcium dependent chloride channels (CLCA) of airway epithelial cells play an important role in the regulation of mucus production \[24,25\]. The expression of human CLCA1 (hCLCA1) is increased in patients with asthma \[26\] and COPD \[27\] in which mucus was excessively produced in the airway. Similarly, the expression of calcium dependent chloride channel 3 (Clca3), the mouse homolog of hCLCA1, markedly increased and was closely correlated with up-regulation of MUC5AC during mucus overproduction in the airway of mice \[28,29\], and we thus investigated whether Clca3 can be also induced by \(\text{PA-LPS}\), and regulated by duox1 during \(\text{PA-LPS}\) induced MUC5AC expression. Furthermore, because \(\text{PA-LPS}\)-induced MUC5AC production is often accompanied by the increased airway inflammation, we investigated whether Duox1 may also regulate the number of total inflammatory cells and neutrophils in BALF of mice treated with \(\text{PA-LPS}\).

To test our hypothesis, we investigated whether Duox1 is induced by \(\text{PA-LPS}\) in A549 cells, primary mouse tracheal epithelial cells (mTECS) and lung tissues of mice respectively, and Nox inhibitors diphenyleneiodonium chloride (DPI) inhibits \(\text{PA-LPS}\)-induced Duox1, MUC5AC and Clca3 expression in vitro and in vivo. The roles of Duox1 and Duox2 in \(\text{PA-LPS}\)-induced MUC5AC expression were also further verified by knockdown of Duox1 and Duox2 in A549 cells using small interfering RNA (siRNA). In the present study, we demonstrated for the first time that Duox1 is partly involved in \(\text{PA-LPS}\)-induced MUC5AC and Clca3 expression.

**Materials and Methods**

**Materials**

A549 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Diphenyleneiodonium chloride (DPI) and dimethylthiourea (DMTU) were from Calbiochem. \(\text{PA-LPS}\) from serotype 10 was from Sigma.

**Animals**

8–10 week-old C57BL/6 mice were purchased from the Experimental Animal Center of Zhejiang University, animal protocols and procedures were approved by the Ethical Committee for Animal Studies at Zhejiang University, China. Mice were once administered \(\text{PA-LPS}\) (100 \(\mu\)g/50 \(\mu\)L) by tracheal cannula. After 30 minutes of \(\text{PA-LPS}\) administration, mice were injected (intraperitoneal injection, i.p) with DPI (1 mg/kg) or DMTU (1 mg per mice) once a day, and sacrificed after 6 days. The BALF of mice was collected to count numbers of inflammatory cells. The lungs were fixed in 10% buffered formalin and stained with Alcian blue/periodic acid-Schiff (AB/PAS) and Hematoxylin & Eosin (H&E) staining.

**Primay Mouse Tracheal Epithelial Cells Culture**

Mouse trachea was isolated from C57BL/6 mice under sterile conditions, and digested with 10 mL 0.15% Pronase solution overnight at 4°C. Then tracheal epithelial cells were harvested and submerged-cultured with mTECS proliferation medium (DMEM/F12 basic media add HEPES, glutamine solution, NaHCO3, heat-inactivated FBS, Retinoic acid, Insulin, Epidermal growth factor.
solution, bovine pituitary extract, Transferrin) in transwell plates (Corning, NY) for 10–14 days. When cells were confluent, the medium in apical side was removed and air-liquid interface (ALI) culture began. After 1-week of ALI culture, cells were stimulated by adding PA-LPS (2.5 μg/mL) and DPI (5 μM) in the basal wells for 24 hours.

A549 Cells Culture

A549 cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum. Before experiments, confluent A549 cells were serum-starved for 24 h to maintain low basal levels of MUC5AC expression.

Real-time PCR

Total RNA was isolated from cells and mice lung tissues using TRIzol Reagent (Invitrogen) according to the manufacturer’s instruction. For RT-PCR, cDNA was generated by reverse transcription using 2 μg total RNA. The expression levels of mClca3 and MUC5AC mRNA were determined by quantitative real-time PCR using the SYBR Green system (Takara) on a spectrophotometric thermal cycler (iCycler; Bio-Rad). The PCR primers are as follows: human MUC5AC: forward: GGACTTCAATATCCAGCTACGC, reverse: CAGCTCAACAACATGCCATC; mouse Muc5ac: forward: GGACTTCAATATCCAGCTACGC, reverse: GGACTTCAATATCCAGCTACGC; human Duox1: forward:
CCTGGCCTCTAGCATG GACAC, reverse: CTGCACCTCC-CACGAAATG; mouse Duox1: forward: GCGATTTGATG-GATGGTAT, reverse: TAGGCAGGTAGGGTTCTTT; human Duox2: forward: AAGTTCAAGCAGTACAAGCGAT, reverse: TAGGCACGGTC TGCAAACAG; mouse Clca3: forward: ACTAAGGTGGCCTACCTCCAA, forward: GGAGGTGA-CAGTCAAGGTGAGA.

RNAi

Human Duox1 and Duox2 siRNAs were purchased from Ambion (Austin, TX). SiRNA was transfected into A549 cells by using Lipofectamine 2000 (Invitrogen, #11668019).

Immunohistochemistry

Cells were incubated with mouse monoclonal antibody to MUC5AC (clone 45 M1, 1:100) for 1 h followed by 10 min in biotinylated rabbit anti-mouse antibody (1:300) and another 10 min in streptavidin–biotin horseradish peroxidase (1:50). Cells were developed for 2 min with diaminobenzidine as chromogen substrate (DAKO Ltd.), counterstained with hematoxylin, and mounted in a xylene-based mountant (BDH-Merck, UK).

H$_2$O$_2$ Measurements

Cells were treated with PA-LPS (10 µg/mL) for 2 h, H$_2$O$_2$ production in the cell supernatants was measured by using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen) according to the manufacturer’s instructions.

ELISA

After reaching confluence and being serum starved for 24 h, cells were stimulated with PA-LPS (10 µg/mL) for 4 h. Cell supernatants were collected and concentrated 10-fold using an Ultracel YM-3 Centrifugal Filter Device (Millipore), and the levels of TGF-β in concentrated cell supernatants were quantified using ELISA kits (R&D Systems Inc, Minneapolis, MN).

Statistical Analysis

Data are presented as mean±SD (n = 3). ANOVA was used to determine statistically significant differences (P<0.05).

Figure 6. Role of ROS in PA-LPS-induced Muc5ac production in vivo. A: DMTU (1 mg per mouse) significantly blocked PA-LPS-induced Muc5ac mRNA and mucin expression in the lung of mice; B: DMTU (1 mg per mouse) inhibited PA-LPS-induced mucin expression in the lung of mice (PAS ×400). #P<0.05 compared with control, *P<0.01 compared with PA-LPS. doi:10.1371/journal.pone.0063945.g006
Figure 7. Effects of DPI on PA-LPS-induced Duox1, Muc5ac and Clca3 production in vivo. A–C: DPI (1 mg/kg) blocked PA-LPS-induced Duox1, Clca3 and Muc5ac mRNA expression in the lung of mice; D: DPI (1 mg/kg) inhibited PA-LPS-induced mucin production in the lung of mice (PAS ×400). #P<0.05 compared with control, *P<0.01 compared with PA-LPS.
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Figure 8. DPI reduced PA-LPS-induced inflammatory cells in BALF and lung tissues of mice. A–B: DPI (1 mg/kg) significantly inhibited PA-LPS-induced neutrophils and total cells in BALF of mice. C: DPI (1 mg/kg) significantly reduced PA-LPS-induced inflammatory cells in lung tissues of mice (H&E ×400). #P<0.05 compared with PBS, *P<0.01 compared with PA-LPS.
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Results

**PA-LPS Induced Duox1 Expression in Primary Mouse Trachea Epithelial Cells and in Mouse Lungs**

As an initial approach to explore the possible role of Duox1 in mediating PA-LPS induced mucus production, the induction of Duox1 by PA-LPS in A549 cells, mTECs and mouse lung tissues was analyzed. As expected, Duox1 mRNA was markedly increased in A549 cells, mTECs and mouse lungs treated with PA-LPS (Figures 1A–1C), though there was no dose-dependent effect for such an induction. These data suggested that Duox1 might positively regulate the mucus production in context of PA-LPS treatment.

**Suppression of PA-LPS-induced Muc5ac and mClca3 Expression by DPI through Decreasing the Expression of Duox1 in Primary Mouse Trachea Epithelial Cells**

To clarify the role of Duox1 in PA-LPS-induced mucus production, we utilized an NADPH oxidase inhibitor DPI which is known to inhibit the function of Duox1 [17], as genetic approaches are difficult to be used in primary epithelial cultures. As shown in Figures 2A–2C, DPI (2.5 μM) significantly inhibited the PA-LPS (2.5 μg/ml) induced mRNA transcripts of Duox1, and also reduced the expression of Muc5ac and Clca3 mRNA, both of which are well recognized markers of goblet cell hyperplasia and mucus hyper-production. These results showed that DPI inhibited PA-LPS-induced-Muc5ac and Clca3 expression partly through decreasing the expression of Duox1 in primary mTECs.

**Duox1 Small Interfering RNA Inhibited PA-LPS-induced MUC5AC Expression in A549 Cells**

To further confirm whether Duox1 is required for PA-LPS-induced MUC5AC expression in airway epithelial cells, we examined the effect of Duox1 knockdown on PA-LPS-induced MUC5AC expression in A549 cells by real-time PCR and immunohistochemistry. As shown in Figure 3A, Duox1 siRNA(100 nM) significantly inhibited Duox1 mRNA expression in A549 cells, and knockdown of Duox1 (100 nM Duox1 siRNA) significantly reduced PA-LPS-induced expression of MUC5AC mRNA in A549 cells (decreased by 44% compared with control, P<0.05) (Figure 3B). Furthermore, using mouse monoclonal antibody to MUC5AC (clone 45 M1, 1:100), we confirmed that 100 nM Duox1 siRNA markedly inhibited MUC5AC protein expression in A549 cells (Figure 3C). These results suggested that PA-LPS-induced MUC5AC expression was Duox1-dependent in airway epithelial cells.

**Duox2 Small Interfering RNA Exerted no Considerable Effects on PA-LPS-induced MUC5AC expression in A549 Cells**

To investigate whether Duox2 is also involved PA-LPS-induced MUC5AC expression, we transfected Duox2 siRNA into A549 cells and showed that Duox2 siRNA could not significantly inhibit PA-LPS-induced MUC5AC expression (Figure 4A–4B). Furthermore, we showed that Duox1 siRNA transfection had no obvious effects on Duox2 expression (Figure 4C). These data supported that Duox2 function is different from Duox1 in airway epithelial cells, especially in term of mucin regulation.

**Duox1 is Required for PA-LPS-induced ROS and TGF-α Production in A549 Cells**

On the basis of our previous finding showing the involvement of the ROS-TGF-α cascade in PA-LPS-induced MUC5AC expression [18], we further investigated whether Duox1 mediates the production of ROS and TGF-α. As shown in Figure 5A and 5B, 100 nM Duox1 siRNA significantly reduced PA-LPS-induced ROS and TGF-alpha production in A549 cells. Taken together, these data demonstrated that Duox1 is involved PA-LPS-induced MUC5AC expression via a ROS-TGF-α-dependent mechanism.

**Effects of DMTU and DPI on PA-LPS-induced Muc5ac and mClca3 Production in vivo**

We have demonstrated both ROS and Duox1 play important roles in PA-LPS-induced MUC5AC expression in vitro, whether same effects could exist in vivo have yet to be addressed. We previously showed that ROS scavenger DMTU significantly decreased PA-LPS-induced MUC5AC production in NCI-H292 cells [18]. Here we further showed that DMTU (1 mg per mouse) significantly blocked PA-LPS-induced Muc5ac mRNA expression in the lung tissues of mice (Figure 6A), and also inhibited mucin over-production as demonstrated by Alcian blue/periodic acid-Schiff (AB/PAS) staining (Figure 6B). As shown in Figure 2A–2C, DPI significantly suppressed PA-LPS-induced Duox1, Muc5ac and mClca3 expression in mTECs. Similarly, DPI (1 mg/kg) also inhibited PA-LPS-induced Duox1, Clca3 and Muc5ac mRNA, and Muc5ac mucin production in mouse lung tissues (Figure 7A–7D). These data demonstrated that Duox1 is partly involved in PA-LPS-induced up-regulation of Muc5ac and Clca3 in vivo.

**DPI Reduced PA-LPS-induced Inflammatory Cells in BALF and Lung Tissues of Mice**

PA-LPS-induced airway mucin over-production is usually accompanied by an increase in inflammatory cells, especially neutrophils. To determine the effects of Duox1 on PA-LPS-induced inflammatory cells in lung of mice, we further examined neutrophils and total inflammatory cells in BALF and lung tissues of mice treated by PA-LPS. As shown in figure 8A and 8B, DPI significantly reduced PA-LPS-induced neutrophils and total cells in BALF of mice, and also reduced inflammatory cells in lung tissues demonstrated by H&E staining (8C), thereby suggesting that Duox1 may be involved in the regulation of PA-LPS-induced airway inflammation in mice.

**Discussion**

To the best of our knowledge, this is the first study to demonstrate that PA-LPS-induced up-regulation of MUC5AC and Clca3 is partly through Duox1 in vitro and in vivo. Here we showed Duox1 is induced by PA-LPS both in vitro and in vivo, and DPI (specific inhibitors of Nox) significantly inhibited PA-LPS-induced up-regulation of MUC5AC and Clca3 partly through decreasing Duox1 expression in mTECs and lung tissues of mice. In addition, we also confirmed that knockdown of Duox1 markedly inhibited PA-LPS-induced MUC5AC expression via a ROS-TGF-α cascade in A549 cells. Furthermore, we demonstrated that DPI significantly inhibited PA-LPS-induced increase in total inflammatory cells and neutrophils accumulated in BALF and lung tissues of mice. On the basis of our previous data that ROS scavengers DMTU reduced PA-LPS-induced MUC5AC production in NCI-H292 cells, we further confirmed that DMTU significantly inhibited PA-LPS-induced Muc5ac in vivo.
Initially identified and cloned from the thyroid gland, Duox are known to be expressed on the surface of ciliated airway epithelial cells [30], and Duox1 is specifically expressed in large airways [31], and its expression is 5-fold higher than that of Duox2 in normal airway epithelium [23]. Harper et al [23] found Duox1 mRNA was specially but moderately increased [by approximately four fold] by Th2 cytokines IL-4 and IL-13. Boots et al [32] showed LPS (10 μg/mL) significantly increased Duox1 mRNA expression in immortalized human bronchial epithelial (HBE1) cells. In this study we showed Duox1 mRNA was up-regulated by approximately 5-fold in vitro and 2.5-fold in vivo by PA-LPS. Interestingly Rada B et al [33] showed *P. pyocyana* produced by *Pseudomonas aeruginosa* inhibited Duox1 activation induced by Th2 cytokines in primary normal human bronchial cells and NCI-H292 cells, suggesting the regulation of Duox1 is different in various microenvironments. Rigotto et al demonstrated activation of Duox1 is Ca²⁺ dependent [34], and whether PA-LPS also activates Duox1 via Ca²⁺ signaling needs to be further investigated.

Human Duox1 and Duox2 are highly similar trans-membrane proteins, while they have distinct function in airway epithelium. Duox2 is mainly involved in responses to infection and inflammation, whereas Duox1 plays an important role in defense and mucus production [17]. Nadel et al [17] confirmed PMA and neutrophil elastase-induced MUC5AC expression was Duox1-dependent in vitro. In this study we found for the first time that PA-LPS-induced MUC5AC production is partly through Duox1, not Duox2, thus providing supportive evidence for the role of Duox1 in mucus production in airway, and supporting that Duox2 function is different from Duox1 in airway epithelial cells, especially in term of mucus regulation. It should be noted that it is the first time to demonstrate that Duox1 was involved in mucus regulation. It is the first time to demonstrate that Duox1 was involved in mucus production in airway, and supporting that Duox2 in normal nasal epithelial (NHNE) cells, and independent of Duox1. Thus Duox1 may be more specific for the bronchial epithelial cells in the regulation of MUC5AC expression. On the other hand, in agreement with our previous data in vitro, we showed DMTU significantly inhibit PA-LPS-induced MUC5AC production in vivo, further suggesting that ROS play important roles in PA-LPS-induced MUC5AC expression.

Clca3 is another known marker of goblet cells in mice airway, and its expression has significantly correlated with MUC5AC. We thank Mr Yonfia Zhu for his help in the pathological manipulation.

**Conclusions**

In summary, we firstly demonstrated that PA-LPS-induced MUC5AC and Clca3 expression are partly through Duox1 in vitro and in vivo, and provide supportive evidence for Duox1 as a potential target in treatments of mucin over-production diseases.

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**Author Contributions**

Conceived and designed the experiments: WL FY HS. Performed the experiments: WL FY HZ XL YW CC. Analyzed the data: WL FY ZC HS. Contributed reagents/materials/analysis tools: NZ ZC JL. Wrote the paper: WL FY HS.

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