



IL-1 β upregulates Muc5ac expression via NF- κ B-induced HIF-1 α in asthma



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ABSTRACT

The manifest and important feature in respiratory diseases, including asthma and COPD (chronic obstructive pulmonary disease), is the increased numbers and hypersecretion of goblet cells and overexpression of mucins, especially Muc5ac. Many proinflammatory cytokines play important roles in goblet cell metaplasia and overproduction of Muc5ac. However, the effect of IL-1 β on Muc5ac expression in asthma remains unknown. Here, we detected the correlation between IL-1 β and Muc5ac in asthma patients and further explored the mechanism of IL-1 β -induced Muc5ac overexpression. Our results showed that Muc5ac and IL-1 β were up-regulated in 41 patients with asthma and that Muc5ac overexpression was related with IL-1 β in asthma ($R^2 = 0.668$, $p < 0.001$). Furthermore, the correlation between IL-1 β and Muc5ac is higher in severe group than that in moderate group. In vitro experiments with normal human bronchial epithelial cells (NHBEs) showed that IL-1 β up-regulated Muc5ac expression in NHBEs in a time- and dosage-dependent manner. Hypoxia-induced HIF-1 α was responsible for Muc5ac expression mediated by IL-1 β . Knocking down HIF-1 α by siRNA decreased Muc5ac expression under hypoxia even in IL-1 β -treated NHBEs cells. Luciferase reporter assay showed that HIF-1 α enhanced Muc5ac promoter activity in HEK293T cells. HIF-1 α could specifically bind to the promoter of Muc5ac by EMSA. The correlation among IL-1 β , HIF-1 α and Muc5ac was observed in patients with asthma. Mechanically, NF- κ B activation was essential to IL-1 β -induced HIF-1 α upregulation via the canonical pathway of NF- κ B. The level of nuclear p65, a subunit of NF- κ B, was obviously increased in NHBEs cells under IL-1 β treatment. IL-1 β did not change either HIF-1 α or Muc5ac expression when inhibiting NF- κ B signaling with Bay11-7082, an inhibitor of NF- κ B. Collectively, we concluded that IL-1 β up-regulated Muc5ac expression via NF- κ B-induced HIF-1 α in asthma and provided a potential therapeutic target for asthma.

1. Introduction

The hallmarks of the airways in many chronic respiratory diseases, such as chronic bronchitis, bronchiectasis and bronchial asthma, are involved in goblet cell hyperplasia and metaplasia, which are associated with mucus hypersecretion. Hypersecretion from numerous goblet cells is regarded as an important contribution to mucus plugging and airway obstruction [1]. Excessive mucus in the airways plays a crucial role in the increase in the frequency and duration of infection, even in the increase in morbidity and mortality in respiratory diseases [2]. Gel-forming mucins include MUC2, MUC5AC, MUC5B, MUC19, and so on. Among these, MUC5AC is the common and major respiratory mucin in goblet cell [3]. The source of mucus is different between the large airways and the small airways. In the large airways, mucus is secreted by goblet cells and submucosal glands, whereas in the small airways mucus is only produced by the goblet cell. Goblet cells can

rapidly produce mucus under certain stimuli in the manner of exocytosis, and then form a mucus layer in the airways. Muc5ac overexpression has been observed both in the large and small airways and plays an important role in the development of asthma. Many proinflammatory cytokines, such as IL-13 and IL-4, up-regulate Muc5ac in lung tissue with asthma via activating transcription factor 6 (STAT6) [4]. These Th2 cytokines induce goblet cell metaplasia/hyperplasia via STAT6, which in turn results in an increase of Muc5ac mRNA and protein levels as expressed by the new airway goblet cells.

Interleukin-1 (IL-1), first described as lymphocyte-activating cytokine, was discovered in the 1970s as a potent inflammatory cytokine [5,6]. IL-1 family was consisted of at least two members. Among them, IL-1 β plays an important role in severe inflammatory diseases, such as the rare cryopyrin-associated periodic syndromes (CAPS). The treatment of anti-IL-1 β can completely control the progression of most of these diseases [7–9]. Increasing evidences have indicated that IL-1 is an

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important regulator in airway hyperresponsiveness and the cytokine production related to Th2 cells [10]. Recently, several studies reported that IL-1-neutralizing drugs reduced airway hyperreactivity and decreased Th2-related cytokines in animal asthma models [11,12]. In addition, IL-1 β mediates MUC5AC gene expression through CREB and/or NF- κ B [13,14]. However, the microenvironment of lung tissue in asthma is complicated. Thus, whether IL-1 β could regulate Muc5ac expression through other mechanism such as hypoxia-induced HIF-1 α in asthma remain largely unknown.

The balance of oxygen supply and demand is required to normal tissue development. However, the balance can be disturbed by numerous physiological and pathophysiological conditions. Under the condition of the reduction in oxygen tension, some tissues usually trigger signaling events and up-regulate several genes to allow for short- and long-term adaptation to hypoxia. The up-regulation of the hypoxia-inducible factor (HIF) family is often initiated under hypoxia, and consequently regulates the transcription of other genes. Although hypoxia can induce HIF expression, HIF-1 α accumulation and transcription can also be triggered under normoxic and inflammatory conditions [15]. Indeed, cytokine-induced up-regulation of HIF-1 demonstrates that HIF-1 is an important line between inflammation and cancer [16,17].

In this study, we evaluated the effect of HIF-1 α on Muc5ac expression in the presence or absence of IL-1 β . Our results suggested that HIF-1 α could enhance Muc5ac overexpression in NHBEs under IL-1 β treatment. Muc5ac was triggered through HIF-1 α binding to Muc5ac promoter. Mechanically, we found that IL-1 β activated NF- κ B signaling and then promoted HIF-1 α expression.

2. Materials and methods

2.1. Subjects

Total of 41 subjects were recruited from Xi'an Ninth People's Hospital. All the asthmatics were divided into moderate group (n = 19) and severe group (n = 22) and were treated according to criteria for asthma [18], exhibiting a provocative concentration of methacholine and inhaled albuterol. All 41 subjects underwent research fiberoptic bronchoscopy with BALF collection and endobronchial biopsy. Bronchial biopsies were taken during diagnostic procedures with a flexible bronchoscope and were immediately formalin-fixed and then paraffin-embedded using standard procedures. All patients were in a stable phase of the disease, with no evidence of exacerbation in the past 4 weeks. Exclusion criteria: significant nonasthma pulmonary disease or other medical problems. 17 control subjects who had no history of respiratory or allergic disease were enrolled. Clinical characteristics of the patients with moderate or severe asthma and control subjects are shown in Table 1. All subjects gave written informed consent and the study

Table 1
Characteristics of the subjects.

	Non-Asthma n = 17	Moderate asthma n = 19	Sever asthma n = 22
Sex(male/female)	8/9	8/11	9/13
Age(years), median	52.5(7.5)	49.4(9.2)	54.6 (8.4)
BMI(Kg/m ²), median	26(6.3)	28(7.8)	30 (8.6)
Smoking history (exsmoker/ current smoker/never smoker)	3/2/12	5/3/11	8/0/14
Atopy(n)	4	7	12
FEV1(%predicted), median	101(5.8)	78.7(6.8)	69.2 (8.4)
ICS dose (mg BDP eq/d)	0	800(0)	1560 (155)
LABA use (%)	0	100	100
Oral corticosteroid use (n)	0	6	10

BMI, body mass index; BDP eq, Beclomethasone dipropionate equivalent; LABA, long-acting β 2-agonist.

was approved by Xi'an Ninth People's Hospital Medical Ethics Committee.

2.2. Cell culture and treatments

Normal human bronchial epithelial cells (NHBEs) (Walkersville, MD, USA) were cultured in bronchial epithelial cell growth medium supplemented with 10% fetal calf serum (FBS) (Gibico, life Technologies, San Diego, CA) at 37 °C in a 5% CO₂ incubator. The medium was exchanged every 48 h, and the cells were grown to confluence for 8 days. Cells were pretreated with 50 mM PD98059 (MAPK inhibitor; Calbiochem, Merck, Germany) or 20 mM LY294002 (Phosphatidylinositol 3-kinase (PI3 K) inhibitor; Calbiochem, Merck, Germany) or 10 mM Bay11-7082 (NF- κ B signaling inhibitor; Calbiochem, Merck, Germany) 30 min before the addition of IL-1 β .

2.3. Hypoxia

Hypoxia and normoxia protocols were performed as described previously [19]. Briefly, Normal human bronchial epithelial cells (NHBEs) (Walkersville, MD, USA) were cultured in bronchial epithelial cell growth medium supplemented with 10% fetal calf serum (FBS) (Gibico, life Technologies, San Diego, CA) at 37 °C in the hypoxic gas mixture (1% O₂, 5% CO₂, and balance N₂). The airtight incubator was kept at 37 °C for preset time periods until harvest.

2.4. Gene silencing with siRNA duplexes

siRNA duplexes sequences against human HIF-1 α and control oligonucleotides were followed: siRNA for HIF-1 α (5'-CUGAUGACCAGCAACUUGA-3'); and siRNA control (5'-AGUUCAACGACCAGUAGUC-3'), which were synthesized and purified in Genray company, China. For preparing the siRNA duplexes, 50 μ M concentrations of antisense and sense oligonucleotides were mixed with annealing buffer [30 mM HEPES (pH 7.0), 100 mM potassium acetate, and 2 mM magnesium acetate], denatured for 1 min at 85 °C, and annealed at 37 °C for 1 h. To confirm the duplexes formation, the electrophoresis through 5% low melting temperature agarose was performed.

For the treatment of cells using siRNA duplexes, cells were plated into 10 cm² cell culture dishes and grown to 30–50% confluence before transfection. The duplexes were diluted to give a final concentration of 20 nM. Then, 3 μ l of X-treme GENE reagent (Roche) were added, then were mixed and incubated at room temperature for 25 min. The cells were then removed any residual serum and incubated with the oligonucleotide duplexes in serum-free conditions for 4 h at 37 °C. Serum was then added back to the culture.

2.5. RNA isolation and real-time PCR

Total RNA extraction from 10 FFPE tissue sections 5 mm thick or cells and cDNA amplification were as described previously [20]. After quantification, 2 μ g RNA was reverse transcribed to cDNA. The target gene HIF-1 α , Muc5ac and β -actin were amplified by real-time PCR. The reaction protocol was as follows: 2 μ l cDNA, 10 μ l 2 $\times$ SYBR Green Mix, 0.2 μ l 10 μ mol/L sense primer, 0.2 μ l 10 μ mol/L antisense primer and 7.6 μ l double distilled water using 7300, a Real-Time PCR machine (ABI). The experiments were repeated by 3 times at least. The Ct mean was calculated and analyzed using $\Delta\Delta$ Ct assay. The oligonucleotides used for IL-1 β : sense: 5'-GCAATGAGGATGACTTGTCTTTG-3'; anti-sense: 5'-CAGAGGTCCAGGTCTGGAA-3'; for HIF-1 α : sense 5'-TGCTAATGCCACCACTACC-3', anti-sense 5'-TGACTCCTTTCTCTGCTCTG-3'; for Muc5ac: sense 5'-TCCGGCCTCATCTTCTTT-3', anti-sense 5'-ACTTGGGCACTGGTGCTG-3'; for control: sense 5'-GACCCCTTCATTGACCTCAAC-3', anti-sense 5'-CATACCAGGAAATG

AGCTTG-3'. All these oligonucleotides were synthesized by Genaray

Company in China.

2.6. Western blot analysis

Protein expression of p65, HIF-1 α and Muc5ac was examined by western blot analysis as described previously [21]. Briefly, NHBECs were washed with PBS and lysed in RIPA buffer. Protein concentration was detected using Bradford reagent (Biorad, Hercules, CA). 40 μ g protein was performed electrophoresis using 10% SDS-PAGE. Then, proteins were electro transferred to a nitrocellulose membrane, blocked with 2% bovine serum albumin (BSA) in TBST, and probed with antibodies against p65 (1:2000 dilution, MILLIPOR), IL-1 β (1:2000 dilution, CST, USA), HIF-1 α (1:3000 dilution, MILLIPOR) and Muc5ac (1:2000 dilution, CST, USA), for overnight at 4 °C. After washing, the blots were exposed to HRP-conjugated secondary antibodies (1:3000 dilution) for 30 min. Then, the aiming protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7. MUC5AC mucin ELISA

NHBECs lysates (50 μ l aliquots) were loaded into 96-well ELISA plates with 50 ml of 2 $\times$ carbonate/bicarbonate buffer. After washing 3 times with PBS, the plates blocked with 2% BSA for 1 h at room temperature. The plates were then incubated with 50 μ l of mouse anti-MUC5AC antibody (1:100; Neomarker, Fremont, CA, USA) for 1 h. The plates were washed 3 times with PBS. 100 μ l peroxidase-conjugated goat anti-mouse immunoglobulin G (1:2500 dilution) was added at each well, followed by incubation for 1 h. Then, the colorimetric reaction was developed by the addition of 100 μ l/well of peroxidase substrate. OD measurements were obtained using an ELISA reader (BIO-TEK Instruments) at 405 nm. The experiments were repeated by 3 times at least.

2.8. Construction of Muc5ac promoter and reporter assays

The preparation of Muc5ac promoter and mutation was carried out as described previously [22]. Briefly, the PCR primers for Muc5ac promoter (sense: 5'-AGGGCAGTACAGAGAACCAC-3'; anti-sense: 5'-G-CTGTGGAGCATGGGGAATG-3') contained an XhoI restriction site in the upstream primer and a HindIII restriction site in the downstream primer, which were synthesized by Generay Company in China. Then PCR was performed using genome DNA as template according to manufactures. This PCR product contained Muc5ac promoter from -150 to +50, including HIF-1 α binding site. This fragment was excised with XhoI and HindIII and subcloned into the plasmid pGL3-Basic to make pGL3 (-150/+50) Muc5ac promoter.

For the reporter assays, transfections were performed using Fugene (Roche) according to the manufacturer's instructions. Briefly, cells were split into 60 mm dishes and were grown to about 50% confluence for 3 days. Then, the mixture of 6 μ l of Fugene and 2 μ g of the reporter plasmid was transfected into cells. Cells were harvested by adding 100 μ l of lysis buffer per dish. The lysis was used for luciferase determinations performing on a TopCount Microplate Scintillation and Luminescence Counter (Packard Instrument Company).

2.9. EMSA

Nuclear proteins were extracted. Oligonucleotides corresponding to -58 to -42 bp of the human Muc5ac promoter were synthesized in Generay Company, Shanghai, China. The complementary sequences 5'-CCACCCACGTGAAGCAC-3' and 5'-GTGCTTCACGTGGGTGG-3', in which underline showed the HIF-1 α binding site, were labeled with DIG-ATP and T4 polynucleotide kinase. Unincorporated DIG-ATP was removed with G-25 Sephadex column (GE, USA). The DNA-binding reaction was done for 30 min at room temperature in a volume of 20 μ l,

containing 5 μ g nuclear protein extract, 2% BSA, 105 cpm of 0.1 mg/mL poly(deoxyinosinic-deoxycytidylic acid) (Sigma), and 5 μ l of 4 $\times$ binding buffer with or without excess of unlabeled competitor oligonucleotide (Promega). Samples were performed to electrophoresis on a 6% polyacrylamide gel for 3 h at 100 V.

2.10. Statistics

Data presented as mean $\pm$ SE and statistical significance was determined by unpaired Student's *t*-test using SPSS software. For analyzing the correlation, we used a two-Pearson's test. The value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. The correlation between IL-1 β and muc5ac in asthma patients

To investigate the function of IL-1 β in asthma, we observed IL-1 β expression in patients with asthma. Compared with subjects without asthma, the patients with asthma significantly emerged IL-1 β up-regulation in mRNA levels. What's more, IL-1 β expression was higher in severe group than that in control and moderate group (Fig. 1A). Meanwhile, Muc5ac expression was analyzed in the patients with asthma. Hypersecretion and overexpression of Muc5ac were detected in all patients with asthma compared with control group (Fig. 1B and C). Importantly, we found that the expression between IL-1 β and Muc5ac had obvious correlation in asthma patients ($R^2 = 0.668$, $p < 0.001$) (Fig. 1D). Intriguingly, the correlation between IL-1 β and Muc5ac is more significant in severe group (Fig. 1F) than that in moderate group (Fig. 1E). Taken together, all these data indicated that IL-1 β expression was upregulated in asthma and was related with Muc5ac, suggesting that IL-1 β might regulate Muc5ac expression in asthma.

3.2. IL-1 β increased Muc5ac expression in NHBEC cells

To determine whether IL-1 β could upregulate Muc5ac expression, we treated NHBEC cells with different dosages of IL-1 β for different durations. As shown in Fig. 2A and B, IL-1 β caused the increased expression and hypersecretion of Muc5ac in NHBEC cells. IL-1 β -induced up-regulation of Muc5ac expression exhibited a dosage-dependent pattern, whereas IL-1 β -induced Muc5ac overexpression showed no difference between 20 ng IL-1 β and 40 ng IL-1 β treatments. Moreover, IL-1 β -induced increase of Muc5ac expression was time-dependent under 20 ng IL-1 β treatment (Fig. 2C and D), although the overexpression of Muc5ac had no change after 1 h when treating with 20 ng IL-1 β . Collectively, all these data suggested that IL-1 β up-regulated Muc5ac expression in NHBEC in a time- and dosage-dependent manner.

3.3. HIF-1 α is required to IL-1 β -induced Muc5ac expression

Previous studies showed that HIF-1 α was an important transcription factor for Muc5ac expression. Thus, we hypothesized that HIF-1 α participated in the Muc5ac overexpression induced by IL-1 β . To test this, we analyzed HIF-1 α expression in NHBEC cell lines stimulated with IL-1 β . As shown in Fig. 3A, IL-1 β increased HIF-1 α expression with Muc5ac in NHBEC cell lines. Next, we asked whether HIF-1 α was required for Muc5ac overexpression induced by IL-1 β . To end this, we inhibited HIF-1 α using siRNA in NHBEC cells. As shown in Fig. 3B, IL-1 β failed to increase the expression and hypersecretion of Muc5ac when transfecting HIF-1 α siRNA into NHBEC cells. Furthermore, we observed the correlation among HIF-1 α , IL-1 β and Muc5ac in severe asthma group, finding that HIF-1 α expression was related with IL-1 β ($R^2 = 0.715$, $p < 0.01$) and with Muc5ac ($R^2 = 0.729$, $p < 0.01$) (Fig. 3C and D). Taken together, HIF-1 α was associated with Muc5ac expression induced by IL-1 β .

Hypoxia is an important factor for HIF-1 α up-regulation. Thus, we

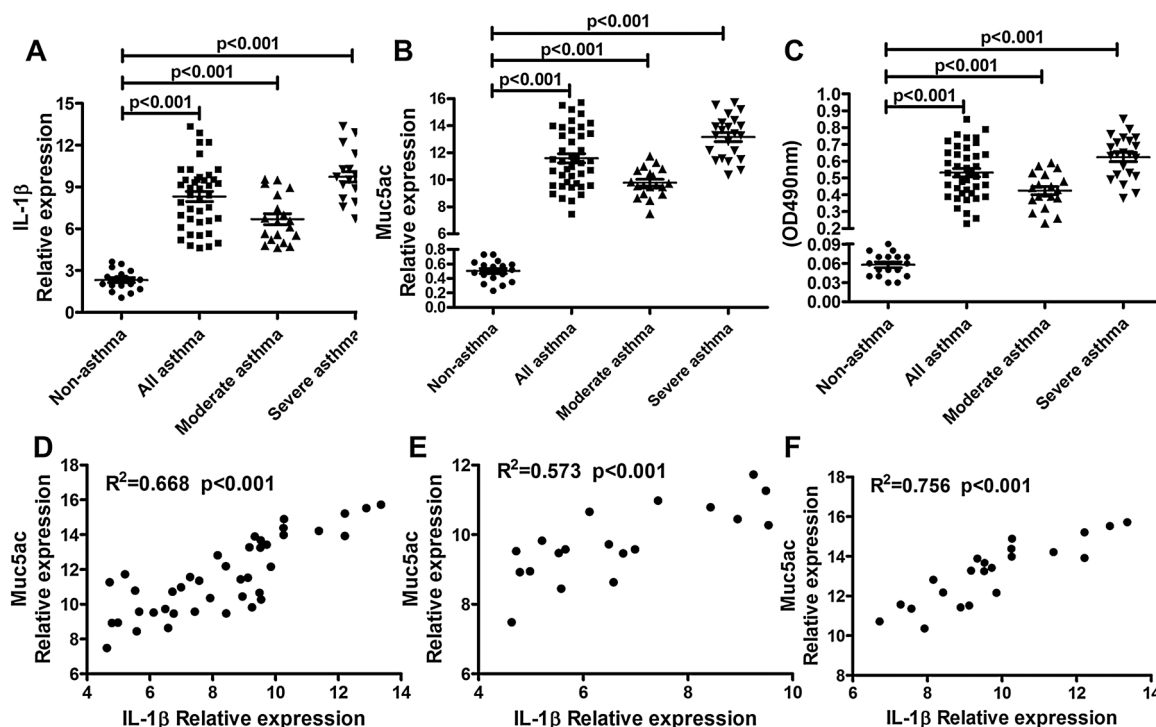


Fig 1. IL-1 β expression was related to Muc5ac in asthma patients. 41 patients with asthma, undergoing respiration biopsy and BALF collection were recruited. The y-axis represents relative expression of IL-1 β expression and Muc5ac expression normalized to β -actin mRNA. IL-1 β and Muc5ac expression in tissue specimens from asthma patients were done. Box plots are shown for IL-1 β expression (A) and Muc5ac expression (B) in asthma patients and lung disease patients without asthma. (C) Secreted Muc5ac protein from 41 patients with asthma was measured. Data are expressed as optical density (OD) at 490 nm. Scatter plots showed the correlation between relative expression of IL-1 β levels and Muc5ac expression obtained from 41 patients with asthma (D), moderate asthma group (E) and severe asthma group (F), respectively. A positive correlation was found by two-Pearson's correlation.

detected Muc5ac expression under hypoxia. Our results showed that hypoxia significantly increased the expression of HIF-1 α and Muc5ac under IL-1 β treatment (Fig. 3E), whereas YC-1, an inhibitor of HIF-1 α , remarkably decreased Muc5ac expression. Together, all these results indicated that HIF-1 α participated in the process that IL-1 β enhanced overproduction and hypersecretion of Muc5ac.

3.4. HIF-1 α induced Muc5ac excessive expression through binding to the promoter

To determine whether HIF-1 α induced by IL-1 β could bind to the Muc5ac promoter, we constructed the Muc5ac promoter with 230 bp length, which contained the HIF-1 α binding site. Then, we treated 293T

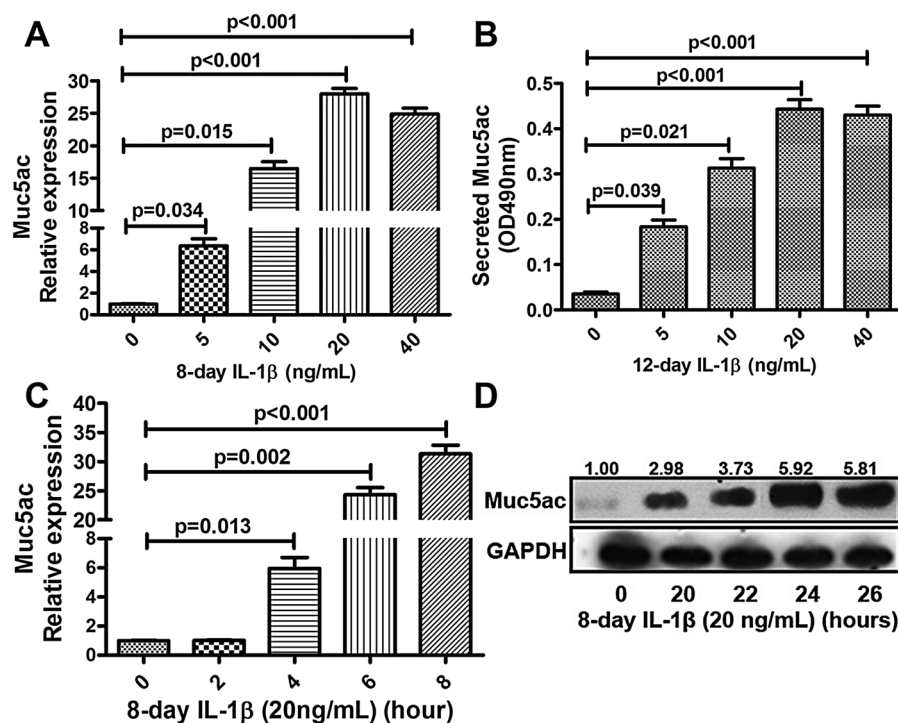


Fig. 2. Effect of IL-1 β on MUC5AC expression. (A) NHBECs were cultured in the presence of different dosages of IL-1 β (0, 5, 10, 20, 40 ng/mL) for 8 days. The mRNA levels of Muc5ac were detected according to the methods. Data are shown by the mean $\pm$ SD of three independent experiments. T-test was used for statistical analysis. (B) NHBECs were cultured 8 days and then treated with different dosages of IL-1 β for another 4 days, and secreted Muc5ac protein was measured. Data are expressed as optical density (OD) at 490 nm. Values are the means $\pm$ SD three independent experiments, $n = 4$. NHBECs were cultured for 8 days, and then were treated with 20 ng IL-1 β for different durations. Secreted Muc5ac mRNA levels (C) and cellular Muc5ac protein levels (D) were measured, respectively. Values represent the mean $\pm$ SD of three independent experiments. T-test was used for statistical analysis.

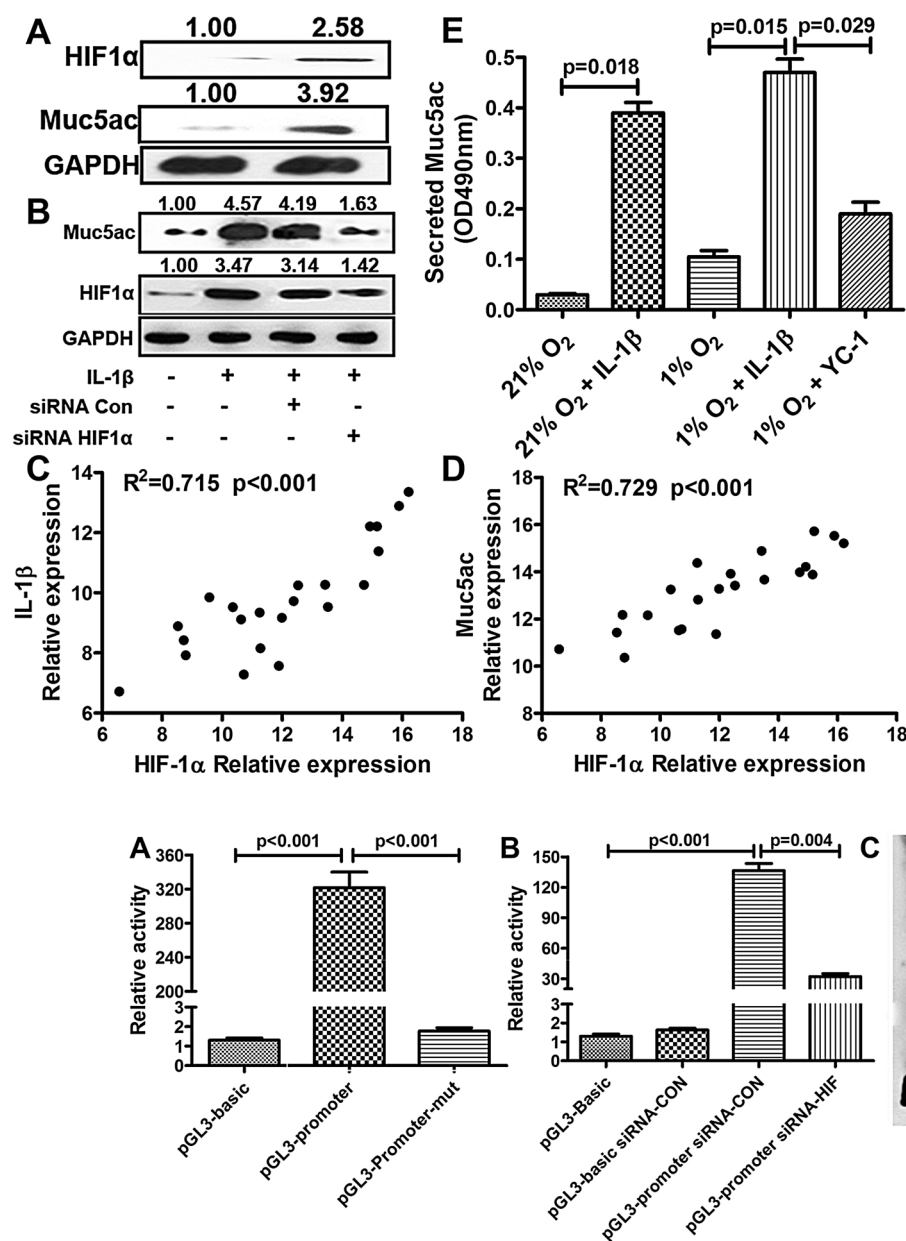


Fig. 3. The effect of HIF-1 α on Muc5ac expression induced by IL-1 β . (A) NHBECs were cultured for 8 days under 20 ng IL-1 β . HIF-1 α on Muc5ac expression were detected. Values represented the relative expression of HIF-1 α on Muc5ac normalized to GAPDH by Image J software. (B) Inhibiting HIF-1 α expression with siRNA in NHBEC cells decreased HIF-1 α on Muc5ac expression induced by IL-1 β . Data are expressed by the relative expression of HIF-1 α on Muc5ac normalized to GAPDH by Image J. The correlation among HIF-1 α , IL-1 β and Muc5ac was analyzed in 22 severe asthma patients. Scatter plots showed the correlation between relative expression of IL-1 β levels and HIF-1 α expression (C) and the correlation between relative expression of Muc5ac levels and HIF-1 α expression (D). A positive correlation was found by Spearman correlation. (E) Hypoxia increases the secreted Muc5ac protein in NHBEC cells in a independent manner of IL-1 β . Data are expressed as optical density (OD) at 490 nm. Values are the means $\pm$ SD three independent experiments.

Fig. 4. HIF-1 α enhances Muc5ac promoter activity under IL-1 β treatment. Muc5ac promoter regions including HIF-1 α element and HIF-1 α element mutation were introduced into the promoter-less pGL3-basic or pGL3-control vector, and the reporter constructs were transiently transfected into 293T under IL-1 β treatment. HIF-1 α induced by IL-1 β could enhance the promoter activity compared with control and mutation (A) and knockdown of HIF-1 α decreased the promoter activity even when treated by IL-1 β . The relative promoter activities are represented as the fold increase versus expression from the activities of renilla plasmid. Values represent the mean $\pm$ SD of three independent experiments. T-test was used for statistical analysis. (C) EMSA confirmed the exact binding site of HIF-1 α in Muc5ac promoter. Cold means cold competitor and mutant means mutant competitor.

cells with either the 230 bp wild-type promoter reporter or the mutation construct in which HIF-1 α binding site was deleted. As shown in Fig. 4A, HIF-1 α induced by IL-1 β could enhance the promoter activity in cells transfected with the plasmid that contained the wild-type Muc5ac promoter. However, in 293T cell lines transfected with HIF-1 α siRNA or with mutation, we failed to detect the promoter activity even when treated by IL-1 β (Fig. 4A and B). These results, coupled with the above, suggested that HIF-1 α induced by IL-1 β could bind to and activate Muc5ac promoter, which in turn lead to an increase the expression and hypersecretion of Muc5ac.

To further test that HIF-1 α could bind to Muc5ac promoter, we performed a gel shift assay. As shown in Fig. 4C, a bond of HIF-1 α binding to Bio-labeled DNA oligonucleotide was detected under treatment with IL-1 β in 293T cells. The binding could be effectively competed away by using excess cold HIF-1 α consensus probe. Taken

together, these results supported the finding that HIF-1 α enhanced Muc5ac overproduction through binding to the promoter.

3.5. IL-1 β upregulated HIF-1 α via activating NF- κ B signaling

Next, we explored the mechanism of IL-1 β upregulating HIF-1 α expression in asthma. Previous studies showed that IL-1 β -induced gene expression was depended on HIF-1 α transcription through PI3K/AKT, MAPK and NF- κ B signaling [23–25]. Thus, we hypothesized that one or of all these signaling was responsible for IL-1 β upregulating HIF-1 α expression in asthma. To end this, we used the inhibitors of these 3 signaling to treat NHBEC cell, respectively. Of these 3 inhibitors, only Bay11-7082, an inhibitor of NF- κ B signaling, decreased the expression of HIF-1 α and Muc5ac under IL-1 β treatment (Fig. 5A), whereas LY294002, an inhibitor of PI3K/AKT, and PD98059, an inhibitor of

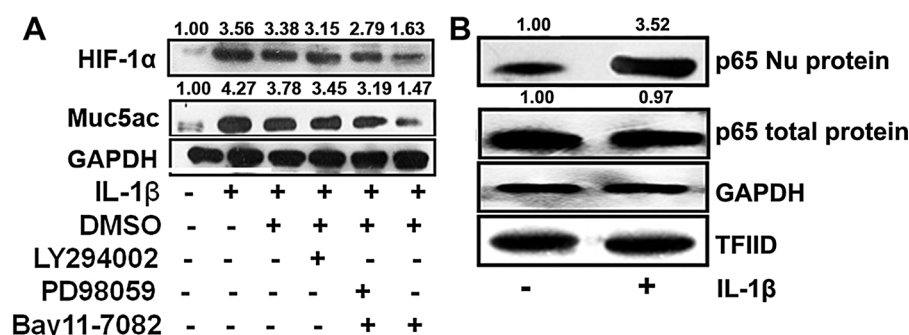


Fig. 5. NF-κB signaling is required to HIF-1α and Muc5ac expression induced by IL-1β. (A) NHBECs were cultured for 8 days under different inhibitor treatment. NF-κB signaling inhibitor, Bay11-7082, decreased the expression of HIF-1α and Muc5ac. (B) IL-1β promotes nuclear translocation of p65 protein in NHBECs. All above data are expressed by the relative expression of HIF-1α on Muc5ac normalized to GAPDH by Image J.

MAPK signaling, had no effect on HIF-1α and Muc5ac expression when treated by IL-1β. Furthermore, we found that nuclear p65 protein level remarkably increased under IL-1β treatment relative to the control (Fig. 5B), suggesting that NF-κB signaling was required to IL-1β up-regulating HIF-1α and Muc5ac expression in asthma. Collectively, these results demonstrated that IL-1β activated NF-κB signaling and made p65 transfer into nuclear, and consequently upregulated HIF-1α expression.

4. Discussions

Mucus overproduction is well-known common feature of the airways in many chronic respiratory diseases, including asthma and COPD. In healthy individuals there are a few mucus-producing cells distal to the trachea, whereas the numbers of goblet cells with excessive mucus production were elevated in asthma and COPD. Secreted mucins can be divided into two subcategories, soluble and gel-forming [26]. Specialized goblet cells in asthma can produce secretory granules, which are consisted of the gel-forming mucin, Muc5ac [27]. Although gel-forming mucins clear from mucosal surfaces by trapping allergens and debris [28], overproduction of mucins, especially Muc5ac, in the airway, increases the frequency and duration of infection and declines the lung function in respiratory disease, including COPD and asthma [2].

Asthma was considered as an immune and allergy-related disease. Many proinflammation cytokines derived from Th2 cells and/or other cells play a crucial role in asthma progression. Among many proinflammation cytokines, IL-1β acts as an immunomodulator in infections derived from pathogenic microorganisms, such as bacteria [29], fungi [30] and viruses [31]. Under these conditions, IL-1β can be stimulated by a variety of host-derived or environmental cellular stressors [32]. Recent findings demonstrate that IL-1β not only induces urticarial rashes in autoinflammatory diseases [33], but also is of importance in other allergy-related diseases, such as bronchial asthma [34]. However, at present, there is still little evidence in the effect of IL-1β on asthma disease. In this study, we observed IL-1β expression in patients with asthma, finding that IL-1β expression with Muc5ac significantly increased. Intriguingly, IL-1β expression was related with Muc5ac expression in patients with asthma ($R^2 = 0.668$, $p < 0.001$). Thus, we reasoned that IL-1β might upregulate Muc5ac expression in asthma. To confirm our hypothesis, we treated NHBEC cells with IL-1β. As expected, our data showed that IL-1β induced Muc5ac expression in a time- and dosage-dependent manner in NHBEC cells. All these results in vivo and in vitro indicated that IL-1β was upregulated and increased Muc5ac overexpression in asthma.

HIF family, a heterodimer, plays an important role in gene regulation as transcription factor. HIF expression initiates the transcription of genes that serve as important regulators in the increase in blood flow through vasodilation, angiogenesis and increase in the blood's oxygen carrying capacity [35,36]. In addition, the activation of HIF can trigger the expression of molecules associated with cellular metabolism and energy production in pathologic process including immune diseases

[37,38]. Previous studies showed that HIF-1α regulated Muc5ac expression in asthma. Thus, we hypothesized that HIF-1α participated in Muc5ac overexpression induced by IL-1β in asthma. As expected, IL-1β upregulated HIF-1α expression and had no increase in Muc5ac expression when HIF-1α siRNA was transfected into NHBEC cells. Importantly, HIF-1α expression in asthma was related with IL-1β ($R^2 = 0.715$, $p < 0.05$) and with Muc5ac ($R^2 = 0.729$, $p < 0.05$) in 22 patients with severe asthma.

Coupled with the function of HIF-1α that it, as an important transcriptional factor, regulates many genes expressions, in current study, our results showed that HIF-1α induced by IL-1β promoted Muc5ac expression. Accordingly, we inferred that HIF-1α enhanced Muc5ac expression through binding to the promoter, which was supported by experiment from Hays W. J. [22], who analyzed the Muc5ac promoter and found a HIF-1α binding site in Muc5ac promoter. If so, we reasoned that HIF-1α could induce Muc5ac expression under IL-1β treatment in asthma. Thus, we evaluated whether HIF-1α activated Muc5ac promoter. As expected, Muc5ac promoter reporter assays showed that HIF-1α could activate Muc5ac promoter, whereas HIF-1α had no effect on the activation of Muc5ac promoter when HIF-1α binding site in Muc5ac promoter was mutated. Besides, EMSA results showed that HIF-1α could bind to Muc5ac promoter. Taken together, our current results suggested that HIF-1α could increase Muc5ac expression through binding Muc5ac promoter.

Mechanically, we found a clue that IL-1β upregulated Muc5ac expression in asthma. HIF-1α played an important role in IL-1β-induced gene expression through PI3K/AKT, MAPK and NF-κB signaling. Using 3 different inhibitors, we found that only NF-κB signaling inhibitor, Bay11-7082, decreased the expression of HIF-1α and Muc5ac under IL-1β treatment, whereas LY294002, an inhibitor of PI3K/AKT, and PD98059, an inhibitor of MAPK signaling, had no effect on HIF-1α and Muc5ac expression when treated by IL-1β. Indeed, IL-1β increased the levels of nuclear p65 protein. Our all these data suggested that NF-κB signaling was required to IL-1β upregulating Muc5ac expression in asthma.

5. Conclusion

In summary, in current study, we investigated the function of IL-1β in asthma and explored the mechanism of IL-1β upregulating Muc5ac expression. Our results indicated that IL-1β and Muc5ac expression was increased in asthma and that IL-1β expression was related to Muc5ac expression. HIF-1α was required to Muc5ac expression induced by IL-1β. Mechanically, IL-1β activated NF-κB signaling and then promoted HIF-1α expression.

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