Lyn kinase represses mucus hypersecretion by regulating IL-13-induced endoplasmic reticulum stress in asthma

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A R T I C L E  I N F O

Article info
Received 11 October 2016
Received in revised form 15 December 2016
Accepted 16 December 2016
Available online 21 December 2016

Keywords:
Mucus secretion
Lyn kinase
Endoplasmic reticulum stress
MUC5AC
Asthma

A B S T R A C T

In asthma, mucus hypersecretion is thought to be a prominent pathological feature associated with widespread mucus plugging. However, the current treatments for mucus hypersecretion are often ineffective or temporary. The potential therapeutic targets of mucus hypersecretion in asthma remain unknown. Here, we show that Lyn is a central effector of endoplasmic reticulum stress (ER stress) and mucus hypersecretion. In Lyn-transgenic mice (Lyn-TG) and wild-type (WT) C57BL/6J mice exposed to ovalbumin (OVA), Lyn overexpression attenuates mucus hypersecretion and ER stress. Interleukin 13 (IL-13) induced MUC5AC expression by enhancing ER stress in vitro. Lyn serves as a negative regulator of IL-13-induced ER stress and MUC5AC expression. We further find that an inhibitor of ER stress, which is likely involved in the PI3K p85α/Akt pathway and Nrf-1 activity, blocked MUC5AC expression in Lyn-knockdown cells. Furthermore, PI3K/Akt signaling is required for IL-13-induced ER stress and MUC5AC expression in airway epithelial cells. The ER stress regulation of MUC5AC expression depends on Nrf-1 in Lyn-knockdown airway epithelial cells. Our studies indicate not only a concept of mucus hypersecretion in asthma that involves Lyn kinase but also an important therapeutic candidate for asthma.

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1. Introduction

Asthma is one of the most common chronic airway inflammatory diseases. Type 2 inflammation is an important molecular mechanism of asthma (Fahy, 2015). The type 2 immune response results in goblet cell metaplasia, mucus hypersecretion, leukocyte infiltration (predominantly eosinophils) and collagen deposition in the airways (Balenga et al., 2013). Previous studies have shown that oxidative stress and ER stress have a direct and pathogenic impact on mucus secretion in asthma. The house dust mite allergen (HDM) induces oxidative injury to the

likely a major cause of mucus plugging in asthma (Bonser et al., 2016). IL-13 induces mucus production and goblet cell hyperplasia in airway epithelial cells. Dexamethasone at therapeutic concentrations did not inhibit the effects of IL-13 on goblet cell differentiation (Kanoh et al., 2011). The activation of MEK1/2, phosphatidylinositol-3 kinase (PI3K), sphingosine kinase 1 (SPHK1), and MAPK14 (p38α MAPK) are critical for IL-13-induced mucus production (Alevy et al., 2012). Transcriptional control of Muc5ac gene expression through regulators such as SPDEF (SAM pointed domain-containing Ets transcription factor), Notch, and Hypoxia Inducible Factor-1 is not currently targetable (Evans et al., 2015). However, further investigation is critical for identifying potential therapeutic targets for mucus hypersecretion in asthma. Endoplasmic reticulum stress (ER stress) causes the activation of activating transcription factor 6 (ATF6), CCAAT/enhancer-binding protein homologous protein (CHOP) and X-box binding protein 1 (XB1) and the phosphorylation of protein kinase-like ER kinase (PERK) (Koh et al., 2013). Previous studies have shown that oxidative stress and ER stress have a direct and pathogenic impact on mucus secretion in asthma.
airway epithelial cells (Li et al., 2012). Asthmatic patients with severe exacerbations exhibit increased oxidative stress damage and NfkB phosphorylation (Lan et al., 2014). HDM induces ER stress in airway epithelial cells (Hoffman et al., 2013). Aspergillus fumigatus is also associated with steroid-resistant eosinophilic allergic lung inflammation via ER stress. Phosphoinositide 3-kinase-δ (PI3K-δ) regulates fungus-induced allergic lung inflammation via endoplasmic reticulum stress (Lee et al., 2016). Furthermore, oxidative stress causes mucin synthesis via the transcription of epidermal growth factor receptor (EGFR), but MUC5AC synthesis is not inhibited by antioxidants (Takeyama et al., 2000). Mucin maturation is achieved by posttranslational modifications initiated in the endoplasmic reticulum (ER) before they traffic to the Golgi. Allergen-induced mucous overproduction is impaired in the absence of ER of specialized ER proteins such as AGR2 and IRE-1 beta (Schroeder et al., 2012, Martino et al., 2013).

Sorc homology-2-containing protein tyrosine phosphatase-2 acts as a negative regulator for H2O2- induced mucous overproduction and hypersecretion in human airway epithelial cells (Song et al., 2013). Lyn kinase, a member of the Src family of tyrosine kinases, modulates mucous production in asthma. Lyn deficiency resulted in the mucous hypersecretion in a mouse model of asthma (Li et al., 2013). Lyn kinase may be one of the most important targets for the treatment of asthma. However, the effects of Lyn kinase on ER stress in asthma are less clear. In this study, we investigated the contribution of Lyn kinase to mucous hypersecretion and ER stress in asthma. Lyn regulated ER stress and MUC5AC expression in a murine model and in our in vitro experiments on airway epithelial cells, resulting in a phenotype associated with PI3K, Akt and Nfkb signals.

2. Methods

2.1. Reagents

The following antibodies for histology and cell staining were purchased from Santa Cruz Biotechnology: anti-MUC5AC (Santa Cruz, CA, sc-16903, AB_649616), anti-phospho-PI3K p85α (Tyr467: Santa Cruz, CA, sc-293115, AB_10844180), anti-PI3K p85α (Santa Cruz, CA, sc-31970, AB_2268186), anti-phospho-Akt1 (Thr308: Santa Cruz, CA, sc-135650, AB_2224730), anti-Akt1 (Santa Cruz, CA, sc-1618, AB_630849), anti-phospho-NfκB p65 (Ser536: Santa Cruz, CA, sc-33020, AB_2179018), anti-NfκB p65 (Santa Cruz, CA, sc-109, AB_632039), anti-Lyn (Santa Cruz, CA, sc-15, AB_2281450), and anti-α-actin (Santa Cruz, CA, sc-130656, AB_2223228). The following antibodies for histology were purchased from Abcam Biotechnology: anti-BIP (Abcam, ab21685, AB_2119834), anti-CHOP (Abcam, ab11419, AB_298023), anti-histone H3 (Abcam, ab1791, AB_302613), and anti-IL-13 (Abcam, ab133353, AB_11517609). Anti-phospho-Lyn (Tyr416: Cell Signaling Technology, #2101, AB_331697) was purchased from Cell Signaling Technology. Anti-IL-13 (R&D Systems, AF-413-NA, and AB_2124173) and IL-13 ELISA reagents (R&D Systems, M1300CB) were purchased from R&D Systems. IL-13 (PeproTech, #200-13), 4-Phenybutyric acid (4-PBA, Sigma-Aldrich, P21005), PI3K Inhibitor PI-103 (Selleck, S1038) were purchased as indicated. A nonsilencing siRNA control and a Lyn-specific siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Human lung tissue

From May 2015 to July 2016, patients with asthma and controls were recruited from the Affiliated Hospital of Southwest Medical University (Sichuan, China). The diagnosis of asthma was based on the asthma guidelines according to our previous descriptions (Li et al., 2013). Human lung tissues were collected according to our previous procedures (Xie et al., 2015). This study was approved by the Southwest Medical University Ethics Committee (Assurance Number: KY2015008).

according to the principle of the Helsinki Declaration II. Participants gave written informed consent to their inclusion in the study.

2.3. Animals and experimental protocol

Transgenic (TG) mice overexpressing Lyn were generated by the Cyagen Biosciences, Inc. (Guangzhou, China). In brief, the Lyn gene was cloned in a transgene vector linking the gene to the EF1α promoter, creating pRP.ExSi-EF1α-mLYN. The transgene DNA was linearized and microinjected into the pronuclei of fertilized eggs of C57BL/6j mice to generate transgenic mice. Stable Lyn-transgenic mice were obtained by crossing founder mice with wild type C57BL/6j mice. The analysis and maintenance for Lyn-transgenic mice was performed in the background of C57BL/6j. There is no differentiation of growth and development in Lyn transgenic mice compared to that in wild type mice in natural lifespan.

Wild type C57BL/6j mice that were 8 to 10 weeks of age and free of murine-specific pathogens were obtained from Tengxin Biotechnology Co., Ltd. (Chongqing, China). The Lyn-transgenic mice and wild type C57BL/6j mice, aged 8–10 weeks, were used for all of the subsequent experiments, which were maintained under specific pathogen-free conditions in the Animal Experimental Center of Southwest Medical University. All animal experiments in this study were approved by and performed in accordance with the guidelines of the Committee of Animal Experiments of Southwest Medical University and the National Institute of Health guidelines on the care and use of animals.

Lyn-TG and WT mice were intraperitoneally sensitized on days 1 and 14 with 20 μg of ovalbumin (OVA) (Sigma-Aldrich,) and 1 ml of normal saline (NS) 2 times per week for duration of 5 weeks, as previously described (Li et al., 2013). Control groups of Lyn-TG mice and WT mice were given NS alone. 4-phenylbutyric acid (4-PBA, 1 g/kg body weight per day; Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) was given to each animal by intragastric administration 2 h before the challenge with OVA and 6 h after the last challenge in OVA-4-PBA mice. Twenty-four hours after the final intranasal challenge, the mice were killed with an intraperitoneal injection of 40 mg/kg ketamine. The trachea was carefully intubated with a 20-gauge catheter, and the lungs were slowly lavaged four times with 1 ml of pre-warmed PBS. The total cell numbers were counted. Smears of the bronchoalveolar lavage (BAL) cells were fixed and stained as described previously to examine cell differentials (Li et al., 2013).

2.4. Histology

The lungs of mice were filled intratracheally with 10% neutral-buffered formalin and then removed. Specimens were embedded in paraffin. For histological examination, sections (5 μm) of the lung specimens were stained with standard hematoxylin-eosin staining (H&E) methods and periodic acid–Schiff (PAS) reagent. The stained slides were analyzed with a DM4000 Leica light microscope (Leica, Germany). Histological review was performed in a blinded fashion by two independent pulmonary observers. The severity of peribronchial and perivascular inflammation was scored using previously described methods. Each bronchus observed was scored from 0 to 3, with approximately 10 areas scored in total (Xie et al., 2015).

2.5. Cell culture, viral infection and transfection

Human airway epithelial cells (16HBE) were obtained from American Type Culture Collection (ATCC; Manassas, USA) and kept in our lab. 16HBEs were used in vitro according to previous report (Sweerus et al., 2016). 16HBEs were seeded in culture dishes with DMEM culture medium containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO2 and grown to 70% confluence. The plV.ExBl.P/Puro–EF1α–IRES-
eGFP/pLV.Des3d/Puro vector was constructed and validated in our lab (data not shown). The lentiviral vector was transfected into human embryo kidney cells (293 T cells) to create infectious lentiviral vector-containing particles. After the 16HBE cells reached 85% confluence, the medium was replaced with serum-free DMEM culture medium. The lentiviral vector expressing Lyn was used to transfect 16HBE cells in the 6-well plates (MOI = 20). For Lyn knockdown, cells were transfected with Lyn small interfering RNA (Lyn siRNA, 20 μM) using Lipofectamine 2000 according to the manufacturer's instructions. Lyn expression was analyzed by Western blotting and immunofluorescence using an SP5 Leica confocal microscope (Leica, Germany). The medium was then replaced with a new medium containing PBS, PI-103 (10 ng/ml) or 4-PBA (10 mM). After 2 h of treatment with the inhibitor PI-103 (10 ng/ml) or 4-PBA, the medium containing IL-13 (10 ng/ml) was added to incubate overnight (24 h) at 37 °C.

2.6. Immunohistochemical staining for Lyn, IL-13, BIP, CHOP, MUC5AC and NFκB

For immunohistochemical staining of Lyn and IL-13, sections (5 μm) of the lung specimens embedded in paraffin were stained using Envision detection kit (Dako, K5007) with Lyn and IL-13 antibody. The stained slides were analyzed with a DM4000 Leica light microscope (Leica, Germany). Immunofluorescence staining was done as described previously (Li et al., 2012, 2013, Xie et al., 2015). Frozen lung tissues embedded in optimal cutting temperature (OCT) compound were sectioned, and immunohistochemical staining was performed on glass slides using standard histological methods. Tissues or cells were fixed with ice-cold methanol and permeabilized in PBS containing 0.25% Triton X-100 for 10 min at room temperature and washed 3 times with PBS. Nonspecific binding was blocked for 1 h with 10% BSA (Sigma-Aldrich) in PBS containing 0.05% Tween 20. Specimens were then incubated with antibodies against IL-13, BIP (Immunoglobulin heavy chain binding protein), CHOP, Muc5ac, and NFκB. FITC-conjugated or TRITC-conjugated secondary Abs was used to probe the primary Abs. After the specimens were washed, nuclei were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen). The specimens were analyzed by immunofluorescence using an SP5 Leica confocal microscope with Leica Application Suite Software (Version number: 14.0.0.162, Leica, Germany). Human lung tissue from asthmatic patients and healthy subjects were kept in our lab. Biopsy specimens were collected as described previously (Xie et al., 2015). A mouse isotype control (Abcam, ab18428), a rabbit isotype control (Abcam, ab27478) or a goat isotype control (Santa Cruz, sc-2028) was replaced with antibodies against IL-13, BIP, MUCSAC and NFκB.

2.7. RNA isolation and real-time PCR

Total RNA was isolated from lung tissue using the TRIzol RNA Reagent (Invitrogen, Thermo Scientific, USA). RNA was quantified using an Epoch multi-volume spectrophotometer system (Biotech, USA). The total RNA preparation was reverse-transcribed using PrimeScript ™ RT Master Mix (Takara, China). Quantitative real-time RT-PCR analysis was performed using SYBR Advantage qPCR Premix (Clontech, USA). The Δcycle threshold method was used to calculate the relative differences in mRNA levels in the Light Cycler 480 Multiple Plate Analysis Software (Roche Diagnostics, USA). The primer sequences were as follows: Gapdh primer (sense, 5′-AAGAAGGTGGTGAAGCAGG-3′; antisense, 5′-GAAGGTGGAAGTGGGAGT-3′; Muc5ac primer (sense, 5′-TCTACACCTCGTCTTCT-3′; antisense, 5′-GACTACACCCTCTGACAC-3′).

2.8. Lung tissue homogenization and cytosolic or nuclear protein extraction

Lung tissue or cells were homogenized with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) with an optional protease inhibitor cocktail (Roche or Fisher scientific). For cytosolic or nuclear protein extraction, cells were collected and washed twice with PBS before nuclear and cytosolic fractionation. Nuclear and cytoplasmatic fractionation was carried out with a nuclear extract kit (Cat: 40410, active motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Each separated protein fraction was analyzed by Western blot.

2.9. Measurement of cytokine levels

Protein concentrations were quantified using an Epoch multi-volume spectrophotometer system (Biotech, USA). The IL-13 levels were determined in triplicate in the total lung lysates from each animal using an ELISA according to the manufacturer's instructions. The lower detection limit is 7.8 pg/ml.

2.10. Western blot analysis

Protein concentrations were determined by an Epoch multi-volume spectrophotometer system (Biotech, USA). Samples (20 μg/lane) were loaded into an SDS-PAGE gel. After electrophoresis at 100 V for 90 min, proteins were transferred to a microporous polyvinylidene difluoride (PVDF) membrane with 100 μm current for 2 h using a wet transfer method. Nonspecific sites were blocked with 5% skim milk in Tris-buffered saline/Tween 20 for 1 h. The blots were then incubated overnight at 4 °C with antibodies against phospho-PI3K p85α, PI3K p85α, phospho-Akt1, Akt1, phospho-NFκB p65 (Ser536), NFκB p65, Lyn, β-actin, BIP, CHOP, histone H3, and phospho-Lyn (Tyr416). Anti-rabbit, anti-mouse, or anti-rat horseradish peroxidase-conjugated IgG secondary antibodies (Cell Signaling Technologies) were used to detect antibody binding, and the bands were visualized using the Pierce ECL Western Blotting kit (Pierce Biotechnology, USA).

2.11. Statistical analysis

All statistical analyses were done using the SPSS 17.0 software (Chicago, IL, USA). All data are presented as the mean ± s.d. Differences among multiple groups were analyzed using a one-way ANOVA. Significant differences between two groups were analyzed by using the Tukey-Kramer post-test or Dunnett’s T3. Statistical significance was defined as a P value < 0.05.

3. Results

3.1. Lyn ameliorated airway inflammation and ER stress in allergic airway inflammation disease

Previous studies have shown that ER stress may be critical for the pathogenesis of bronchial asthma, especially the steroid-resistant neutrophilic asthma (Kim et al., 2013). Nonetheless, we found that Lyn modulates the development of airway remodeling (Li et al., 2013), raising the question of whether Lyn may affect the ER stress implicated in inflammation: the features of inflammation and increased inflammatory disease. In present studies, immunohistochemical staining showed that Lyn-TG mice showed increased expression of Lyn the airway epithelium, alveolar epithelial cell and inflammatory cells in lung compared with WT mice (Supplementary Fig. S1 online). OVA-induced the characteristic features of allergic airway inflammation: an increased number of total cells, neutrophils and eosinophils in the bronchoalveolar lavage fluid (BALF); lung inflammation, and increased inflammatory index. In OVA-induced Lyn-TG mice, the features of...
allergic airway inflammation were mitigated: the number of total cells, neutrophils and eosinophils in the BALF were decreased, and lung inflammation and the inflammatory index were both decreased. As expected, 4-PBA also significantly suppressed the characteristic features of OVA-induced chronic airway inflammation shown above in positive control mice compared with that in normal WT mice (Fig. 1b–f).

To investigate the effect of Lyn on ER stress in airway epithelial cells in the mouse model of allergic airway inflammation, the localization of ER stress markers expressed in lung tissue was determined by immunofluorescence staining. Confocal microscope analyses revealed that OVA induced the features of ER stress, increasing the expression of CHOP and BIP in the lung tissue of OVA-induced WT mice. The expression of BIP and CHOP predominantly occurred in the cytoplasmic areas of the airway epithelium in OVA-induced WT mice. OVA-induced mice exhibited lower fluorescence intensities corresponding to BIP and CHOP in the lung tissue than OVA-induced WT mice (Fig. 1h–i). 4-PBA also substantially suppressed BIP and CHOP in OVA-induced WT mice (Fig. 1g). The immunofluorescence intensities of BIP and CHOP in 4-PBA-treated positive control WT mice were significantly decreased by 39.2 (14.63/40.13) and 34.5 (19.66/57.03) percent, respectively, compared with those in OVA-induced WT mice (Fig. 1h–i, P < 0.05). Taken together, these results indicated that Lyn overexpression repressed the OVA-

Fig. 1. Lyn regulated airway inflammation and ER stress in allergic airway inflammation disease. (a) Experimental design for the sensitization and challenge of WT mice and Lyn-TG mice with OVA. (b–d) Total cell numbers and cell differentials in the BALF were determined. (e–f) Representative H&E–stained sections of the lungs of WT mice and Lyn-TG mice (original magnification: 200×). The degree of peribronchial and perivascular inflammation was scored (Kruskal-Wallis test). (g) Representative immunohistochemical staining of BIP or CHOP in the lung tissue of WT mice and Lyn-TG mice. (h–i) Quantitation of the fluorescence intensity of BIP or CHOP per micrometer in 10 random fields. All data are presented as the mean ± s.d. (n = 8 each group, one-way ANOVA with Tukey-Kramer post-test.)
induced chronic airway inflammation and ER stress in mice exposed to OVA. The amelioration of airway inflammation caused by Lyn overexpression may be associated with the inhibition of ER stress.

3.2. Lyn inhibited mucus hypersecretion and ER stress associated with PI3K, Akt and NFκB

The role of ER stress in airway mucus production has been established. Our previous studies have found that Lyn modulates mucus hypersecretion and goblet cell hyperplasia in mice exposed to house dust mite allergen (Li et al., 2013). However, it remains unclear whether Lyn-regulated mucus secretion is associated with ER stress.

To investigate whether Lyn suppresses ER stress or unfolded protein response (UPR) in the airway epithelium, 4-PBA was evaluated as a positive control for ER stress in WT mice exposed to OVA. We observed a direct correlation among goblet cell hyperplasia, Muc5ac expression and ER stress. 4-PBA significantly suppresses goblet cell hyperplasia and Muc5ac expression in positive control WT mice exposed to OVA. Similarly, we observed that Lyn kinase regulates mucin secretion and Muc5ac expression in Lyn-TG mice exposed to OVA (Fig. 2a). The number of goblet cells present in the airway epithelium of Lyn-TG mice exposed to OVA decreased by 60.2% (39.33/65.33) compared with that in WT mice exposed to OVA (Fig. 2b, P < 0.001). The immunofluorescence intensities of Muc5ac in the airway epithelium of Lyn-TG mice

![Fig. 2. Lyn inhibited mucus hypersecretion and the ER stress-associated pathway in allergic airway inflammation disease. (a) Representative PAS staining of the lung tissue of WT mice and Lyn-TG mice. Representative immunohistochemical staining of Muc5ac in the lung tissue of WT mice and Lyn-TG mice (original magnification: 200×). (b–c) The goblet cell percentage was quantified in 10 random fields. The fluorescence intensity of Muc5ac was quantified per micrometer in 10 random fields. (d) RT-PCR of Muc5ac mRNA expression. (e) Representative Western blots of phospho-PI3K p85α, PI3K p85α, phospho-Akt1, Akt1, phospho-NFκB p65 (Ser536), NFκB p65, Lyn, β-actin, BIP, CHOP, and phospho-Lyn (Tyr416) in the lung tissue. (f–i) Relative changes in the density of phospho-Lyn and β-actin, phospho-PI3K p85α and PI3K p85α, phospho-Akt1 and Akt1, and phospho-NFκB p65 (Ser536) and NFκB p65, as measured by Western blot. Data are representative of three experiments. Bars represent the mean ± s.d (n = 8 each group, one-way ANOVA with Tukey-Kramer post-test).]
exposed to OVA were reduced by 59.0% (35.57/60.29) compared to those in WT mice exposed to OVA (Fig. 2c, P = 0.027). The mRNA levels of Muc5ac in the lung tissue were measured by quantitative real-time PCR. We observed a robust decrease in Muc5ac transcript levels in the Lyn-TG mice exposed to OVA compared with WT mice exposed to OVA (61.1% decrease (2.00/3.27); Fig. 2d; P = 0.007).

Previous studies showed that PI3K-α regulates Aspergillus fumigatus-induced allergic lung inflammation through ER stress (Lee et al., 2016). To investigate whether Lyn is involved in ER stress in the pathogenesis of bronchial asthma, we also measured the levels of ER stress markers and PI3K/Akt/Nf-κB in the lung tissue of WT mice and Lyn-TG mice exposed to OVA. The levels of BIP and CHOP showed a 1.8-fold (1.32/0.72) and 1.7-fold (1.25/0.72) increase, respectively, in mice exposed to OVA compared to those in mice exposed to normal saline (NS). We further observed a robust decrease in the levels of BIP and CHOP in Lyn-TG mice exposed to OVA compared to those in WT mice exposed to OVA (Fig. 2e), suggesting a link between Lyn and ER stress in asthma.

ER stress-mediated Nf-κB activation and PI3K pathway-regulated mucus hypersecretion are associated with bronchial asthma pathogenesis (Lee et al., 2016, Kim et al., 2013). In positive control mice, 4-PBA also suppressed the phosphorylation levels of PI3K p85, Akt and Nf-κB in the lung tissue of WT mice exposed to OVA compared with the levels in PBS-treated controls (Fig. 2g, h). The phosphorylation levels of PI3K p85, Akt and Nf-κB in Lyn-TG mice exposed to OVA compared with those in WT mice exposed to OVA (Fig. 2g), were reduced by 59.0% (35.57/60.29) compared to those in WT mice exposed to OVA (Fig. 2h).

IL-4, IL-5 and IL-13 is related to disease progression in patients with a type 2 allergic phenotype (Trivedi et al., 2016). Its signaling promotes the differentiation of goblet cells. IL-13 severely impairs mucus transport by airway epithelial cells (Tyner et al., 2006, Yan et al., 2014, Bonser et al., 2016) and induces ROS formation and ER stress in airway smooth muscle (Delmotte and Sieck, 2015). Consistent with previous results showing an increase in IL-13 in the lungs of mice exposed to OVA, we found that Lyn and 4-PBA suppressed IL-13 in mice exposed to OVA (Fig. 3a, b). Aside from IL-13, we have also found that Lyn over-expression impacted on IL-4 and IL-5 in asthma mice models. Lyn over-expression and 4-PBA suppressed the levels of IL-4 and IL-5 in mice exposed to OVA (Supplementary Fig. S4a and S4b online).

To investigate the involvement of Lyn in IL-13-induced ER stress and MUC5AC expression in airway epithelial cells, we measured the levels of MUC5AC, BIP and CHOP in airway epithelial cell lines (16HBE) using lentiviral Lyn overexpression vectors. Similar results were seen for the IL-13 levels, and Lyn over-expression also reduced IL-13-induced MUC5AC expression in 16HBE cells (Fig. 3c, f). Notably, confocal microscopic analyses revealed that BIP and CHOP were predominantly in the cytoplasm of 16HBE cells after addition of IL-13 (Fig. 3d–e). The overexpression of Lyn in 16HBE cells led to a dramatic decrease in BIP and CHOP after the addition of IL-13 compared with control cells (16HBE cells with stable GPX expression). The fluorescence intensities of BIP and CHOP decreased significantly by 39.7% (18.91/47.59) and 24.4% (8.07/33.12), respectively, in Lyn+/+ cells (16HBE cells with stable Lyn and GPX expression) after the addition of IL-13 compared with their intensities in control cells (Fig. 3f). The BIP and CHOP protein levels in 16HBE cells increased markedly after the addition of IL-13 in comparison with their levels after the addition of PBS (Fig. 3g, h). Supporting the idea that IL-13-induced ER stress may be involved in mucus overproduction. Thus, Lyn plays a critical role in IL-13-induced ER stress and MUC5AC expression in airway epithelial cells.

In animal experiments, our studies found that Lyn-mediated ER stress and mucus hypersecretion were involved in the PI3K p85/Akt pathways in the mouse model of allergic lung inflammation. To investigate how Lyn mediates ER stress and mucus hypersecretion in airway epithelial cells, we measured the levels of PI3K p85/Akt/Nf-κB in lung tissue using Western blotting. A marked decrease was observed in the phosphorylation levels of PI3K p85, Akt and Nf-κB in Lyn+/+ mice compared to the levels in control cells (Fig. 3g–h). Collectively, these studies suggest that IL-13-mediated PI3K p85/Akt/Nf-κB pathways can be inhibited by overexpression of Lyn.

3.4. Lyn repressed IL-13-induced Nf-κB activity in airway epithelial cells

Previous studies suggested that the regulation of MUC5AC expression is intimately linked to Nf-κB-based transcriptional mechanisms that are mediated by the binding of Nf-κB to a specific area within the MUC5AC promoter (Koeppen et al., 2013). We found that the nuclear translocation of Nf-κB was critical for MUC5AC induction (Xie et al., 2015). To examine the effects of IL-13 on Nf-κB nuclear translocation in airway epithelial cells, the expression localization of Nf-κB was determined by immunofluorescence staining. Confocal microscope analysis revealed that IL-13 induced significant Nf-κB nuclear translocation (Fig. 4a). In addition, Western blot analysis revealed that the Nf-κB p65 levels in cytoplasmic extracts of cells was significantly decreased in IL-13-induced control cells compared with the levels in PBS-treated control cells. The Nf-κB p65 levels in nuclear extracts of cells was significantly increased in IL-13-induced control cells compared with the levels in PBS-treated control cells. The levels of Nf-κB p65 in the nuclear extracts of cells increased approximately 2.57-fold (1.57/0.61) in IL-13-induced control cells compared with the levels in PBS-treated controls (Fig. 4b, c, P = 0.001). The results indicated that IL-13 can induce Nf-κB activity.

To investigate the effect of Lyn in IL-13-induced Nf-κB activity in airway epithelial cells, we next examined Nf-κB activity in cells infected with the Lyn-expressing lentivirus or control vector. Interestingly, confocal microscope analysis also revealed that Lyn substantially reduced the increase in Nf-κB levels in the nucleus of IL-13-induced Lyn+/+ cells compared with the Nf-κB levels in IL-13-induced control cells (Fig. 4a). The Nf-κB p65 levels in the nuclear extracts of cells were significantly decreased in IL-13-induced Lyn+/+ cells compared with the levels in IL-13-induced control cells. The levels of Nf-κB p65 in the nuclear extracts of cells showed a decrease of approximately 43.1% (0.68/1.57) in the IL-13-induced Lyn+/+ cells compared with the levels in IL-13-induced control cells (Fig. 4b, c, P = 0.003). Together, these data suggest that the overexpression of Lyn suppresses IL-13-induced Nf-κB activity in airway epithelial cells.

3.5. ER stress regulated the activity of Nf-κB, not PI3K p85/Akt in Lyn-knockdown airway epithelial cells

In our present studies, we found that 4-PBA decreased mucus secretion in OVA-exposed WT mice (Fig. 2a), raising the question of whether
4-PBA also affects MUC5AC in Lyn-knockdown cells (transfected with Lyn siRNA). To further examine the effects of 4-PBA on MUC5AC in Lyn siRNA-transfected 16HBE cells in the presence of IL-13, we observed MUC5AC and the expression of BIP and CHOP in Lyn siRNA-transfected 16HBE cells in the presence of IL-13 (Fig. 5a–c). The fluorescence intensity of MUC5AC increased by approximately 0.33-fold (14.80/44.95) in Lyn-knockdown cells compared to its intensity in siCTRL cells in the presence of IL-13. We observed a direct correlation between the fluorescence intensities of MUC5AC and 4-PBA, detecting an approximately 48.2% (28.81/59.76) decrease in the fluorescence intensity of MUC5AC in Lyn-knockdown cells treated with 4-PBA compared to the intensity in cells not exposed to 4-PBA (Fig. 5a, d, P = 0.016). Furthermore, 4-PBA suppresses IL-13-induced nuclear translocation of NFκB in Lyn-knockdown cells (Fig. 5a).

Consistent with our previous results, 4-PBA decreased the expression of BIP and CHOP in WT mice after exposure to OVA. 4-PBA decreased the levels of phospho-PI3K p85α and phospho-NFκB p65 in Lyn-knockdown cells compared to their levels in WT mice in the presence of OVA (Fig. 3).
(Ser536) in WT mice exposed to OVA (Supplementary Fig. s2 online). To confirm these observations, we analyzed the expression of BIP and CHOP in Lyn-knockdown airway epithelial cells in the presence of IL-13. 4-PBA decreased the expression of BIP and CHOP in siCTRL cells and Lyn-knockdown cells in the presence of IL-13 (Fig. 5c). Furthermore, we analyzed the activity of PI3K p85, Akt and NfκB in Lyn-knockdown airway epithelial cells in the presence of IL-13. Lyn deficiency increased the phosphorylation of PI3K and Akt in the presence of IL-13. Lyn-knockdown 16HBE cells showed approximately 0.31-fold (0.17/0.54) and 0.44-fold (0.28/0.64) increases in the levels of p-PI3K p85/PI3K p85 and p-Akt/Akt, respectively, compared with the levels in siCTRL cells in the presence of IL-13 (Fig. 5f, g). Interestingly, 4-PBA did not significantly inhibit the levels of p-PI3K p85/PI3K p85 and p-Akt/Akt compared with the levels in cells that were not treated with 4-PBA in Lyn-knockdown cells in the presence of IL-13 (Fig. 5f–g, P = 0.045 and P = 0.037). Consistently, 4-PBA did not significantly inhibit the levels of p-NFκB/NFκB compared to levels in cells that were not treated with 4-PBA in Lyn-knockdown cells in the presence of IL-13 (Fig. 5f, g, P = 0.023).

3.6. The PI3K p85/Akt pathway was critical for IL-13-induced ER stress and MUC5AC in Lyn-knockdown cells

PI-103, a PI3K inhibitor, has been shown to have a direct inhibitory effect on PI3K and Akt (Bagci-Onder et al., 2011) and is considered one of the most potent compounds in blocking the phosphorylation of Akt (Fan et al., 2006). To determine whether PI3K directly influences IL-13-induced ER stress and mucus overproduction in asthma, we examined the effect of PI-103 on ER stress markers in airway epithelial cells. Consistent with our previous results, the fluorescence intensities of BIP and CHOP increased by approximately 0.27-fold (1.13/1.48) and 0.42-fold (18.72/44.81), respectively, in IL-13-treated Lyn-knockdown cells compared to that in siCTRL cells (Fig. 6a, P = 0.024 and P = 0.032). The data demonstrate that Lyn knockdown increased the IL-13-induced ER stress in airway epithelial cells. Furthermore, the fluorescence intensities of BIP and CHOP showed robust decreases of approximately 52.7% (28.00/53.17) and 56.6% (35.94/63.53), respectively, in IL-13-treated Lyn siRNA cells in the presence of PI-103 compared with the intensities in the PBS controls (Fig. 6a–c, P = 0.008 and P = 0.002).

To further evaluate the role of PI3K in mucus overproduction in asthma, we also examined the effect of PI-103 on MUC5AC in airway epithelial cells. In the presence of PI-103, confocal microscope analyses revealed that Lyn-knockdown 16HBE cells showed an approximately 52.1% (31.18/59.78) decrease in the fluorescence intensity of MUC5AC after addition of IL-13 (Fig. 6a, d, P = 0.005). Hence, PI-103 significantly decreases the fluorescence intensities of NfκB p65 in Lyn-knockdown 16HBE cells after the addition of IL-13.

Western blot analysis showed that protein levels of BIP and CHOP were higher in IL-13-treated Lyn siRNA-transfected 16HBE cells than in control siRNA-transfected cells. PI-103 significantly inhibited the increase in the BIP and CHOP protein levels in Lyn-knockdown 16HBE cells after the addition of IL-13 (Fig. 6e). Consistent with these observations, Lyn knockdown significantly increased the phosphorylation of Akt in Lyn siRNA-transfected 16HBE cells compared with that in control siRNA-transfected cells after the addition of IL-13. PI-103 blocked the phosphorylation of Akt after the addition of IL-13 to Lyn siRNA-transfected 16HBE cells. After the addition of IL-13, the phosphorylation levels of Akt showed an approximately 66.8% (0.79/1.18) decrease in the presence of PI-103 in Lyn siRNA-transfected 16HBE cells compared to those in cells grown in the absence of PI-103 (Fig. 6e, g, P = 0.001). Lyn knockdown also significantly increased the IL-13-induced phosphorylation of NfκB p65 in Lyn siRNA-transfected 16HBE cells compared to that in control siRNA-transfected cells after the addition of IL-13. In the presence of PI-103, the phosphorylation levels of NfκB p65 showed an approximately 56.1% (0.54/0.97) decrease in Lyn siRNA-transfected 16HBE cells after the addition of IL-13 (Fig. 6f, P = 0.003). These results indicate that PI3K/Akt signaling is required for IL-13-induced ER stress and mucus overproduction in airway epithelial cells. Thus, the ER stress regulation of mucus secretion depends on NfκB in Lyn-knockdown airway epithelial cells.

To explore the potential clinical value of this newly revealed molecular pathway, we analyzed the expression levels of BIP, CHOP and IL-13 in the lung tissue of asthmatic patients. Patients with asthma exhibited infiltration of inflammatory cells (Fig. 7a). We also found that immunohistochemical staining for BIP, CHOP and IL-13 in the lung tissue of
Asthmatic patients was significantly increased compared to that in healthy subjects (Fig. 7b–g). It was negative that the images from isotype staining of human lung showed in Supplementary Fig. s5 online. Fig. 7h illustrates a model delineating the role of Lyn in IL-13-induced mucus secretion related to the PI3K p85/Akt/ER stress/NFκB pathways. Lyn overexpression decreased IL-13-induced mucus secretion via the PI3K p85/Akt/ER stress/NFκB pathways.

4. Discussion

Asthma is an increasingly common chronic disease that is characterized by underlying airway inflammation and airflow obstruction and is caused by the interaction of various genetic and environmental factors. The prevalence of asthma has continued to rise over the past 50 years. The epithelium plays an important role in asthma and shows evidence of damage along with the presence of more mucus-producing cells in asthma (Grainge and Davies, 2013). Previous studies have shown that house dust mite-induced oxidative stress damage and DNA repair modulate asthma-associated pathophysiology (Chan et al., 2016). ER stress is generally defined by the unfolded protein response (UPR). UPR regulates the expression of numerous genes that maintain homeostasis in the ER (Walter and Ron, 2011). 4-PBA reduces ER stress by acting as a chemical chaperone (Chen et al., 2016). Molecular chaperones participate in the folding process of protein through recognizing, retaining and targeting mis-folded proteins for their eventual degradative pathway (Welch and Brown, 1996). Chemical chaperone 4-PBA prevents the aggregation of mis-folded proteins and corrects folding of proteins to get their right conformation, which is commonly used to alleviate ER stress (Wang et al., 2016). 4-PBA is also a histone deacetylase inhibitor. Previous studies indicated that 4-PBA mediated ER stress-induced neuronal cell death through the chemical chaperone activity rather than inhibition of histone deacetylase (Mimori et al., 2013). Chemical chaperone 4-PBA prevented cigarette smoke-induced mucociliary clearance disruption (Jochems et al., 2015). ER stress has also been implicated in the pathogenesis of bronchial asthma through the modulation of NFκB. Inflammation of human bronchial epithelia
and activates the ER stress transducer inositol-requiring enzyme 1 (IRE1) α, resulting in airway epithelial mucin production (Martino et al., 2013). 4-PBA alleviated ER stress in the murine model of asthma (Kim et al., 2013). Here, we show that 4-PBA significantly suppressed the characteristic features of OVA-induced chronic airway inflammation and mucus secretion in positive control mice. Our data reveal that 4-PBA decreased goblet cell hyperplasia and Muc5ac expression was accompanied by inhibition of ER stress, suggesting that ER stress was important in mediating these effects. However, it remains possible that the decreased mucus secretion is caused by other activities of 4-PBA such as HDAC inhibition in our murine model of asthma.

Lyn kinase participates in the downstream signaling pathways of a variety of receptors on the plasma membrane, serving as a signaling platform under oxidative stress (Matsuda et al., 2006). In our previous studies, we observed chronic house dust mite exposure-induced mucus hypersecretion in Lyn-knockout mice (Li et al., 2013). In the current study, we tested the hypothesis that Lyn plays a role in mucus hypersecretion by regulating ER stress in asthma. Notably, using Lyn transgenic mice, the expression of Lyn increased in the airway epithelium, alveolar epithelial cell and inflammatory cells. The over-expression of Lyn in epithelial cells and inflammatory cells may be associated with the phenotype of allergen-challenged Lyn-TG mice. We confirmed that Lyn overexpression suppressed OVA-induced chronic airway inflammation and mucus hypersecretion in mice exposed to OVA. Lyn ameliorated the pathological features of asthma but also showed evidence of decreased ER stress in an asthmatic murine model. We further note that decreased mucus secretion is associated with decreased ER stress in Lyn-overexpressing mice despite chronic exposure to OVA.

Lyn tyrosine kinase regulates PI3K activity (Kannan et al., 2008). The activity of Lyn is crucial for the activation of PI3K. Lyn deficiency increased basal and inducible PI3K activity in the hyper-responsive phenotype of B cells (Xu et al., 2012). PI3K/Akt signaling at the mitochondria-associated endoplasmic reticulum membranes regulate mitochondrial physiology (Betz et al., 2013). The present study indicates that PI3K p85 and Akt are phosphorylated when mice are exposed to OVA. These findings are consistent with previous studies showing...
that PI3K/Akt are critical in regulating allergic lung inflammation (Liu et al., 2015, Bonifazi et al., 2010). We found that phosphorylation of PI3K p85 and Akt was decreased in Lyn-overexpressing mice exposed to OVA and inhibited ER stress in the lungs. ER stress triggers the activation of NF-κB, a key molecule in the onset of inflammation (Cantero-Recasens et al., 2010). Previous studies have indicated that NF-κB-based transcriptional mechanisms modulate the transcriptional regulation of MUC5AC during acute lung injury (Koeppen et al., 2013). In support of this, our results revealed that the enhanced phosphorylation of NF-κB that accompanies an increased mucus hypersecretion in response to ER stress in the lung was suppressed in Lyn-TG mice exposed to OVA.

Previous studies confirmed that interleukin-13 directly affects epithelial cells to cause airway hyper-reactivity and mucus overproduction in asthma (Kuperman et al., 2002). IL-13 has been shown to significantly enhance the ER stress activated by lipopolysaccharide in microglia (Liu et al., 2010). ER stress is able to influence inflammatory signaling through modulation of the JNK and NF-κB pathways. Altered UPR signaling is differentially sensitive to the various biological effects of IL-4 and IL-13 (Arensdorf and Rutkowski, 2013). IL-13 is highly expressed in Lyn-deficient mast cells. Lyn, as a negative regulator of allergen-stimulated bone marrow-derived mast cells, regulated the expression of the IL-13 gene (Hernandez-Hansen et al., 2005). The binding of IL-13 triggers the recruitment of several molecules, such as PI3K, Akt, and Src kinase, and enables activation of PI3K (Bartolome et al., 2015). We further found that Lyn overexpression significantly repressed IL-13 expression in the lungs of mice exposed to OVA. Interestingly, we also found that in the presence of IL-13, Lyn overexpression can help prevent ER stress and decrease MUC5AC expression. Lyn deficiency enhanced ER stress and increased MUC5AC expression in airway epithelial cells. We further found that treatment with 4-PBA blocked MUC5AC expression in Lyn-knockdown cells, which are implicated in the PI3K p85/Akt pathway and NF-κB activity. Our studies provide further insight into Lyn-mediated effects on mucus production through ER stress.

ER stress leads to the activation of the PI3K/Akt signaling pathway. Akt promotes NF-κB activity by increasing NF-κB nuclear translocation and the transcription of its target genes (Hamanaka et al., 2009,
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