Biochemical and Biophysical Research Communications 446 (2014) 49–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Interleukin-13-induced MUC5AC expression is regulated by a PI3K–NFAT3 pathway in mouse tracheal epithelial cells

Fugui Yan [a,1], Wen Li [a,1], Hongbin Zhou [a], Yinfang Wu [a], Songmin Ying [a], Zhihua Chen [a], Huahao Shen [a,b,*]

[a] Department of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang, China
[b] State Key Lab. of Respiratory Disease (SKLRS), China

ARTICLE INFO

Article history:
Received 3 February 2014
Available online 26 February 2014

Keywords:
Interleukin-13
MUC5AC
Nuclear factor of activated T cells 3
Phosphoinositide 3-kinase

ABSTRACT

Interleukin-13 (IL-13) plays a critical role in asthma mucus overproduction, while the mechanisms underlying this process are not fully elucidated. Previous studies showed that nuclear factor of activated T cells (NFAT) is involved in the pathogenesis of asthma, but whether it can directly regulate IL-13-induced mucus (particularly MUC5AC) production is still not clear. Here we showed that IL-13 specifically induced NFAT3 activation through promoting its dephosphorylation in air–liquid interface (ALI) cultures of mouse tracheal epithelial cells (mTECs). Furthermore, both Cyclosporin A (CsA, a specific NFAT inhibitor) and LY294002 (a Phosphoinositide 3-kinase (PI3K) inhibitor) significantly blocked IL-13-induced MUC5AC mRNA and protein production through the inhibition of NFAT3 activity. We also confirmed that CsA could not influence the forkhead Box A2 (Foxa2) and mouse calcium dependent chloride channel 3 (mClca3) expression in IL-13-induced MUC5AC production, which both are known to be important in IL-13-stimulated mucus expression. Our study is the first to demonstrate that the PI3K–NFAT3 pathway is positively involved in IL-13-induced mucus production, and provided novel insights into the molecular mechanism of asthma mucus hypersecretion.

1. Introduction

Airway mucin hypersecretion (particularly MUC5AC and MUC5B) contributes to morbidity and mortality in asthma [1]. Previous studies have demonstrated that interleukin-13 (IL-13) plays a critical role via IL-13 receptor and STAT6 transcription factor [2,3] in asthma mucus overproduction [4–6], while the mechanisms underlying this process still need to be defined.

Nuclear factor of activated T cells (NFAT) is a transcription factor originally identified in T cells, composed of four distinct members that are regulated by the calcium/calcineurin signaling pathway, known as NFAT1 (also called NFATp, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3). Another NFAT member called NFAT5 (TonE-BP) is regulated by hyperosmotic stress. NFAT has been confirmed to be involved in the pathogenesis of asthma [7–9]. Fonseca et al. [8] showed a significant decreased number of mucus-producing cells in the lung of OVA-challenged NFAT1$^{-/-}$ mice, but whether or not NFAT can directly regulate mucus production is still not clear. On the other hand, several transcription factors including forkhead Box A2 (Foxa2) and SAM pointed domain-containing ETS transcription factor (SPDEF) which are involved in lung morphogenesis and respiratory epithelial differentiation play an important role in asthma mucus overproduction [10–11], and Davé et al. have shown that NFAT is also required for perinatal lung maturation and function [12]. Therefore, we hypothesize that NFAT may also participate in regulation of asthma MUC5AC expression.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that mediates various cellular functions, including mitogenesis, survival, motility, and differentiation. PI3K is involved in IL-13-induced increase of goblet cells density [13], and can induce NFAT activation in T cells [14]. Furthermore, Moon et al. [15] showed PI3K is involved in osteoclast differentiation through NFAT2, so we questioned whether a PI3K–NFAT cascade mediated IL-13-induced MUC5AC expression.

As mentioned, Foxa2 has a crucial role in IL-13-induced MUC5AC expression [10], and NFAT4 is a direct activator of Foxa2 genes [12], so we further explored whether NFAT is dependent on foxa2 to function its role. Calcium dependent chloride channels (CLCA) of airway epithelial cells play an important role in the regulation of mucus production, and mouse calcium dependent chloride channel 3 (mClca3, the homolog of human CLCA1) can

* Corresponding author at: Department of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou, 310009, China. Fax: + 86 571 8778 3729.
E-mail address: huahaoshen@163.com (H. Shen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.02.051
0006-291X/

mediate IL-13-induced mucus overproduction [16], we therefore sought to investigate the correlation between NFAT and mClca3 in IL-13-induced MUC5AC expression.

Here we firstly demonstrated that a PI3K–NFAT3 pathway is positively involved in IL-13-induced MUC5AC expression in air–liquid interface (ALI) cultures of mouse tracheal epithelial cells (mTECs), and Cyclosporin A (CsA) could not influence the Foxa2 and mClca3 expressions in IL-13-induced MUC5AC production. These findings may provide new insights into the pathogenesis of asthma mucus overproduction.

2. Materials and methods

2.1. Materials

Recombinant human IL-13 was purchased from R&D, Inc. CsA-was from Sigma, Inc. Antibodies against MUC5AC (45M1), mClca3, Foxa2, p-NFAT1 (Ser274) and p-NFAT2 (Ser259) were from Santa Cruz, Inc. Antibodies against p-NFAT3 (phosphor S168 + S170), p-NFAT4 (phosphor S165), and β-actin were from Abcam, Inc. LY294002 was from Calbiochem, Inc.

2.2. Primay mouse tracheal epithelial cells culture

Primary air–liquid interface cultures of mTECs were established as described previously [17]. Mouse trachea was isolated from 8 to 10 week-old 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 ALI culture began. After 1-week of ALI culture, cells were stimulated by adding IL-13 (10 ng/mL) alone or in combination with CsA or LY294002 in the basal wells for 14 days, and the medium was replaced every other day.

2.3. Real-time PCR

Total RNA was isolated from mTECs with ALI cultures 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 MUC5AC, NFAT1, NFAT2, NFAT3, NFAT4, mClca3, and Foxa2 mRNA were determined by quantitative real-time PCR using the SYBR Green system (Takara) on a spectrofluorometric thermal cycler (iCycler; Bio-Rad). The PCR primers are as follows: mouse MUC5AC: forward, GGACTTCAATATCCAGCTACGC, reverse, GGACTTCAATATCC AGCTACGC; mClca3: forward, ACTAAGGTGGCCTACCTC CAA, reverse, GGAGGTGACAGTCAAGGTGAGA; mouse NFAT1: forward, C CACCACGAGCTATGAGAAGA, reverse, GTCAGCGTTTCGGAGCTTCA ; mouse NFAT2: forward, CAGTGTGA CCGAAGATACCTG G, reverse, TCGAGACTTGATA GGGACCCC; mouse NFAT3: forward, GAGCTGGAATTTAAGCTGGT GT, reverse, CATGGA GGGGTATCCTCTGAG; mouse NFAT4: forward, GTATGGATCTGG ACACTCCTTGT, reverse, CGTCGTTTACCACAGGGAGA; mouse Foxa2: forward, CAT GGGACCTCACCTGAGTC, reverse, CATCGAGT TCATGTT GGCG TA; mouse β-actin: forward, GGCTGTATTCCCCTCCATCG, reverse, CCAGTTGGTAACAA TGCCATGT.

2.4. Western blotting analysis

After stimulation for 14 days, the cells were harvested in cell lysis buffer, separated on 8–10% SDS–PAGE gels and transferred to polyvinylidine difluoride membranes (Pall Life Sciences, Pensacola, FL). The membrane was blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% nonfat milk. After three washes in TBS-T, the membrane was incubated with a 1:1000 dilution of a primary antibody for overnight. After another three washes in TBS-T, the membrane was incubated with 1:2000 dilution of the corresponding secondary antibody for 1 h. The membrane was reacted with enhanced chemiluminescence (ECL, Amersham Biosciences) to visualize to blots.

2.5. Statistical analysis

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

3. Results

3.1. IL-13 induced NFAT3 activation and MUC5AC expression in mTECs

Consistent with previous reports [19], we also demonstrated that the expression of MUC5AC mRNA and protein were significantly increased in ALI cultures of mTECs treated with IL-13 (10 ng/mL) for 14 days (Fig. 1A and B), while only mildly increased in those stimulated by 1 ng/mL of IL-13 for 24 h, 48 h and 14 days as well as 10 ng/mL of IL-13 for 24 h and 48 h respectively. To determine the role of NFAT in IL-13-induced MUC5AC expression, we analyzed NFAT1, NFAT2, NFAT3 and NFAT4 mRNA expression in the ALI cultures of mTECs treated with IL-13 (10 ng/mL) for 24 h, 48 h and 14 days respectively. However, we found no significant up-regulation of all four NFAT mRNA levels (data not shown), which indicated that IL-13 may induce the activation of NFAT proteins and not their expression levels. Next we explored the activation and expression levels of NFAT1, NFAT2, NFAT3 and NFAT4 proteins. The activation process of NFAT is as follows: extra

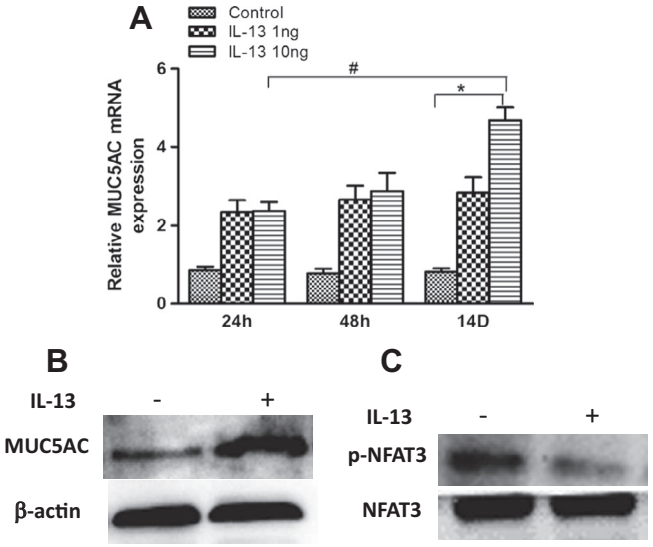

Fig. 1. IL-13 induced NFAT3 activation and MUC5AC expression in mTECs. (A) The expression of MUC5AC mRNA in ALI cultures of mTECs treated with IL-13 (1 ng/mL, 10 ng/mL) for 24 h, 48 h and 14 days. (B) The MUC5AC protein expression in ALI cultures of mTECs treated with IL-13 (10 ng/mL) for 14 days. (C) The phosphorylated NFAT3 and total NFAT3 levels in mTECs treated with IL-13 (10 ng/mL) for 14 days. Data are expressed as means ± SD ($n = 3$). $^{\#}P < 0.05$, $^{*}P < 0.01$.