Asthma is a respiratory inflammatory disease, and nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) is involved in the progression of respiratory diseases. However, the role of NOX4 in asthma remains unclear. In the present study, we aimed to explore the effects of NOX4 on airway remodeling and inflammation. NOX4 expression was measured using immunocytochemistry (IHC), western blot, and real-time PCR (qPCR). Lung tissues were stained using the H&E assay. ELISA was used to examine the levels of airway remodeling-related indicators, and qPCR was used to detect airway inflammatory factors. The results indicated that NOX4 is highly expressed in lung tissues, bronchoalveolar lavage fluid (BALF), and serum of OVA-treated mice. Inhibition of NOX4 alleviated OVA-induced airway remodeling and inflammation. Similarly, TGF-β1 was also upregulated in BALF and serum OVA-induced mice. Inhibition of TGF-β1 signaling also improved airway remodeling and inflammation induced by OVA. Moreover, the downregulation of NOX4 inactivated the TGF-β1-Smad2/3 pathway, and TGF-β1 decreased Smad2/3 expression. Moreover, inhibition of the TGF-β1 was enhanced, while TGF-β1 reversed the effects on airway remodeling and inflammation induced by NOX4 inhibition. Taken together, the downregulation of NOX4 improves airway remodeling and inflammation via inactivation of the TGF-β1-Smad2/3 pathway in asthma mice, suggesting that NOX4 may be a therapeutic target for asthma.
tive therapeutic interventions, ultimately improving the outcomes and quality of life for individuals living with asthma.

Nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) is a member of the NOX family, a key enzyme that leads to the production of reactive oxygen species (ROS) (7). NOX4 is the only subtype of the NOX family that can produce hydrogen peroxide (H₂O₂). It is initially detected to be highly expressed in the kidneys and then found to be expressed in other types of cells, such as endothelial cells, smooth muscle cells, and osteoclasts (8,9). Aberrant expression of NOX4 is associated with numerous diseases, including renal diseases, lung diseases, and cancers (10-12). The role of NOX4 in pulmonary diseases is contradictory. It has both destructive and protective effects on the respiratory tract (11). NOX4 is closely linked to ciliary function, smooth muscle function, and fibroblast differentiation in asthma (13-15). However, the effect of NOX4 on airway remodeling and inflammation of asthma and the underlying mechanism remains unknown.

In the study, we investigated the role of NOX4 in asthma and the underlying mechanisms. We found that NOX4 is highly expressed in OVA-induced mice, which suppression improved airway remodeling and inflammation. Mechanically, the effect of NOX4 is acted through the TGF-β1-Smad2/3 pathway. These findings provided a novel target for asthma therapy.

Materials and Methods

Animals

The animal study was approved by the Ethics Committee of The first affiliated hospital of Bengbu Medical College. BALB/c mice (6-8 weeks old; female; Hunan SJA Laboratory Animal Co., Ltd., Changsha, China) were housed at 22 ± 1°C temperature, 45-55% humidity, and 12 h light/dark cycle SPF conditions. Then the mice were randomly divided into 4 groups: control, OVA, OVA+ DPI, OVA+ SB431542, and OVA+ DPI+ SB431542 groups. The mice in the control and OVA groups only received nasal perfusion with the same amount of normal saline. To establish asthma mice, they were intraperitoneally injected with 0.2 ml of normal saline containing 50 μg ovalbumin (OVA, Aladdin, Shanghai, China) and aluminum hydroxide (1 mg; Sigma-Aldrich, St. Louis, MO, USA) at 0, 7, and 14 days. Starting on day 21, OVA mice were given aerosolization of 1 % OVA normal saline for 30 min every day, continuous for 3 weeks. The mice in the control were injected with 0.2 ml saline at 0, 7, and 14 days intraperitoneally and aerosolized using saline for the same time. To inhibit NOX4, 0.5mg/kg DPI (MedChemExpress, Shanghai, China) was intraperitoneally injected into mice (16). To inhibit TGF-β1 signaling, 0.5 mL of SB431542 (MedChemExpress) at 2 μg/mL was used for nasal inhalation. To increase TGF-β1 expression, 0.1 μg TGF-β1 (Pepro-Tech) was intraperitoneally injected into mice. The mice were sacrificed by intraperitoneal injection with 200 mg/kg pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA). Serum, bronchoalveolar lavage fluid (BALF), and lung tissues were collected for further study.

Immunocytochemistry (IHC) assay

The lung tissues were fixed with formaldehyde for 24 h, embedded in paraffin, making pathological sections. Paraffin sections were dewaxed with xylene and gradient alcohol. Then the sections were incubated with 3% H₂O₂ for 10 min to remove endogenous catalase. After digesting with citric acid buffer, the sections were blocked using normal goat serum. The sections were incubated with primary antibodies (anti-NOX4 (ab109225, 1/500) and anti-TGF-β1 (ab215715, 1/500), Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with secondary antibody (ab150113, 1/500, Abcam, Cambridge, MA, USA) at 37°C for 0.5 h. After washing, the sections were stained with DAB and hematoxylin. The images were visualized under a microscope.

H&E staining assay

Isolated lung tissues were fixed with 4% paraformaldehyde for 24 h and cut into 5 μm sections after paraffin embedding. All sections were stained with Hematoxyline for 10 min and eosin for 1 min. The stained sections were visualized under a microscope. The airway wall area (Wt), the perimeter of the bronchial basement membrane (p bm), and smooth muscle thickness were measured using the Image Pro Plus software.

Western blot

Proteins were extracted with pre-cooled RIPA lysis buffer (Solarbio, Beijing, China). After protein concentration was detected using a BCA Protein Assay Kit (Solarbio, Beijing, China), equal proteins were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5 % non-fat milk for 1 h, incubated with primary antibodies (anti-NOX4: ab109225, 1/2000; anti-TGFBR1: ab2355578, 1/1000; anti-TGF-β1: ab275715, 1/1000; p-Smad2/3: ab202445, 1/1000; anti-β-actin: ab8227, 1/3000; Abcam, Cambridge, MA, USA) overnight at 4°C, and then incubated with the secondary antibody (ab6721, 1/3000; Abcam, Cambridge, MA, USA) for 1 h at room temperature. The bands were visualized using an ECL substrate (Solarbio, Beijing, China). β-actin was used as an internal reference.

Real-time PCR (qPCR)

Total RNA was isolated from lung tissues homogenates using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA was used for reverse-transcribed and qPCR using M-MLV reverse transcriptase (Tiangen, Beijing, China) and SYBR Green (Solarbio, Beijing, China). qPCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as the internal control. The relative expression was calculated using the 2ΔΔCt method. The sequences of the primers were listed as follows: NOX4 F: 5’-TCTG-GCTT-CCTC-CCATGAATGTC-3’ and R: 5’-TCTGGTGGAAA-CTTCTTGTGA-3’; β-actin F5’-AAAGACCTGTACCAGCC AACACAGTGCTGCTGG-3’ and R5’-CGTCATACTTCC TGGTGTCCATAGCACCAC-3’.

ELISA

The expression of TGF-β1, IL-4, IL-13, ET-1, and VEGF in BALF and serum was detected using their specific ELISA kits (Jiancheng, Nanjing, China) following the manufacturer’s protocol. Cysteinyl leukotrienes 1 (CysLT1) and cysteinyl leukotriene receptor 1 (CysLTR1)
levels in lung tissues were also detected using the CysLT1 ELISA kits (Tongwei, Shanghai, China) and the CysLTR1 ELISA kit (Gaining, Shanghai, China) following the manufacturer’s protocol, respectively (17). Lung tissues were homogenized in normal saline. The supernatant was obtained after centrifugation at 1000×g for 10 min. CysLT1 and CysLTR1 levels were detected using the supernatant.

Statistical analysis

Data from three independent experiments were analyzed using GraphPad Prism 7.0 software (La Jolla, CA, USA) and presented as the mean ± standard deviation. Comparisons were analyzed using Student’s t-test and one-way analysis of variance (ANOVA) followed by Tukey’s test. *P < 0.05 was considered statistically significant.

Results

Effects of NOX4 on airway remodeling and inflammation

To evaluate the role of NOX4 in asthma mice, the expression of NOX4 in lung tissues, BALF, and serum was evaluated using IHC staining assay, western blot, and qPCR. As shown in Figure 1A, NOX4 was significantly highly expressed in lung tissues, BALF, and serum, while DPI reduced NOX4 levels. Furthermore, OVA observably induced the increase of inflammatory cells in the airway, Wat/pbm, as well as the smooth muscle thickness, while DPI markedly reversed the increase induced by OVA (Figure 1B). The levels of airway remodeling factors ET-1, IL-4, IL-13, and VEGF were significantly evaluated by OVA in both BALF and serum, while DPI notably reduced their levels (Figure 1C). The number of total cells, eosinophils, neutrophils, lymphocytes, and macrophages increased by OVA, which were markedly abrogated by DPI (Figure 1D). Moreover, OVA significantly elevated CysLT1 and CysLTR1 levels, which were notably abrogated by DPI (Figure 1E).

Effects of TGF-β1 signaling inhibition on airway remodeling and inflammation

As illustrated in Figure 2A, we found that the expression of TGFBR1 was markedly upregulated in BALF and serum of mice in the OVA group, while SB431542 abolished the upregulation, suggesting the TGF-β1 pathway was inactivated. Then, the results of the H&E staining assay showed that the inflammatory cells in the airway, Wat/pbm ratio, and smooth muscle thickness were all significantly increased by OVA, which were significantly reversed by SB431542 (Figure 2B). The levels of ET-1, IL-4, IL-13, and VEGF in BALF and serum were significantly evaluated by OVA, but inhibiting of TGF-β1 signaling markedly abolished the effects induced by OVA (Figure 2C). OVA markedly increased the number of total cells, eosinophils, neutrophils, lymphocytes, and macrophages, while SB431542 significantly reversed the increase (Figure 2D). Additionally, the levels of CysLT1 and CysLTR1 were significantly enhanced in OVA mice, which were notably abrogated in the mice in the OVA+SB431542 group (Figure 2E).

NOX4 is positively related to the TGF-β1-Smad2/3 pathway

The data of the western blot indicated that TGF-β1 and p-Smad2/3 levels were upregulated in OVA mice, while DPI abolished the upregulation (Figure 3A). Furthermore, we found that TGF-β1 treatment increased p-Smad2/3 levels in OVA mice (Figure 3B). Additionally, DPI reversed the upregulation of p-Smad2/3 levels induced by TGF-β1.
SB431542 enhanced the effects of NOX4 on airway remodeling and inflammation

We explored the role of both DPI and SB431542 in OVA mice. Compared with the OVA+DPI group, the expression of NOX4 and TGF-β1 was downregulated in the OVA+DPI+SB431542 group (Figure 4A). The levels of ET-1, IL-4, IL-13, and VEGF induced by OVA were decreased by DPI and further decreased by SB431542 (Figure 4B). Additionally, the number of inflammatory cells, CysLT1, and CysLTR1 levels were all reduced by DPI, which were further reduced by SB431542 (Figure 4C and D).

TGF-β1 treatment reversed the effects of NOX4 on airway remodeling and inflammation

Finally, we performed the rescue experiments. TGF-β1 increased Wat/bpm ratio and the smooth muscle thickness in DPI-treated mice (Figure 5A). TGF-β1 abrogated the levels of airway remodeling factors ET-1, IL-4, IL-13, and VEGF reduced by DPI (Figure 5B). The number of total cells, eosinophils, neutrophils, lymphocytes, and macrophages was reduced by DPI, which was markedly abrogated by TGF-β1 (Figure 5C). Moreover, DPI significantly reduced CysLT1 and CysLTR1 levels in OVX mice, and TGF-β1 markedly rescued the effects induced by DPI (Figure 5D).

Discussion

Herein, we investigated the role of NOX4 in asthma and the underlying mechanism. We found that downregulated NOX4 contributed to alleviating airway remodeling and inflammation. Inactivating the TGF-β1-Smad2/3 pathway enhanced the effects of NOX4 inhibition on airway remodeling and inflammation.

In recent years, the incidence of asthma is increasing, but its occurrence mechanism is not identified. Airway remodeling and airway inflammation are known as two main factors of asthma pathogenesis (18). Airway remodeling is a change in the structure, cellular, and molecular composition of the airway wall and is generally believed to result from long-term airway inflammation (19). The interaction of inflammatory cells such as eosinophils, neutrophils, lymphocytes, and macrophages causes the onset of asthma (20,21). The release of IL-4, IL-5, and IL-13 by immune system cells is an important feature of high Type 2 T helper lymphocytes (Th2) asthma (22). CysLT1 mediates most of the pathological processes of asthma through its receptor CysLTR1, especially inflammation (23). ET-1 is associated with airway remodeling, especially in the absence of obvious Th2 asthma (24). Moreover, VEGF regulates vascular growth in airway remodeling, thus contributing...
to the inflammatory response in Th2-type asthma (25). In this study, we used OVA to induce asthma, we found OVA increased inflammatory cell number, Wt/bpm, smooth muscle cells, IL-4, IL-13, ET-1, VEGF, CysLT1, and CysLTR1 levels. The findings suggested that we successfully established an asthma model, and asthma was associated with airway remodeling and inflammation.

Dysregulation of NOX4 is related to asthma. Through genome-wide microarray analysis and qPCR experiment, NOX4 is identified to be upregulated in airway smooth muscle in asthma, and its expression in COPD is higher than in asthma (14, 26). NOX4 also regulates the function of pulmonary artery smooth muscle cells to influence asthma (27). Additionally, NOX4 is expressed in bronchial epithelial cells, and inhibition of NOX4 eliminates ciliary dysfunction in mice with asthma (13). Herein, we clarified that NOX4 was highly expressed in serum, BALF, and lung tissues of asthma mice. Furthermore, we used DPI to inhibit NOX4 expression. The results showed that DPI reversed the effects on airway remodeling and inflammation induced by OVA, suggesting that downregulated NOX4 improved airway remodeling and inflammation, and further attenuated the progression of asthma.

TGF-β is known to play a crucial role in asthma by regulating inflammation, cell growth, and fibrosis. TGF-β1 is an isoform of TGF-β that participates in airway remodeling (28). The levels of TGF-β1 produced by inflammatory cells are increased in airway lavage fluid (29). The results of bronchial biopsies TGF-β1 is expressed in most eosinophils (30). Additionally, TGF-β1 signaling transduction commonly depends on the Smad pathway. TGF-β1 activates its receptors in a paracrine and autocrine manner, leading to the phosphorylation of the Smad proteins (31). In the context of asthma, the TGF-β1/Smad pathway modulates airway remodeling, inflammation, choroidal neovascularization, and airway smooth muscle cell proliferation (32-34). In addition, NOX4 has been reported to regulate the TGF-β1/Smad pathway in multiple diseases, such as acute kidney injury, lung fibrosis, and atrial fibrosis (35-37). NOX4 promotes TGF-β1-induced ROS production, airway smooth muscle cell growth, and hypertrophy, therefore affecting the pathological process of asthma (38). Herein, we found that SB431542 decreased TGF1 expression, suggesting inhibiting the TGF-β1 pathway in mice with asthma. Moreover, DPI decreased OVA-induced p-Smad2 and p-Smad3 levels, whereas TGF-β1 increased OVA-induced p-Smad2 and p-Smad3 levels. Functionally, SB431542 enhanced the effect on airway remodeling and inflammation induced by DPI, and TGF-β1 reversed the effects induced by DPI. The findings suggested that inhibition of NOX4 expression improved airway remodeling in asthmatic mice by the TGF-β1-Smad2/3 pathway.

The results of this study shed light on the significant role of NOX4 in the pathogenesis of asthma. The findings demonstrate that the levels of NOX4 were elevated in various compartments, including bronchoalveolar lavage fluid (BALF), serum, and lung tissues, in mice with asthma. This upregulation of NOX4 suggests its involvement in the development and progression of asthma. Furthermore, the study reveals that the downregulation of NOX4 has a beneficial impact on airway remodeling and inflammation in the context of asthma. The inhibition of NOX4 activity resulted in improved airway structure and reduced inflammation, indicating its potential as a therapeutic target. Notably, these beneficial effects were mediated through the inactivation of the TGF-β1-Smad2/3 pathway, which is known to play a pivotal role in asthma pathogenesis. The identification of NOX4 as a key player in asthma pathophysiology opens new avenues for the development of targeted therapies. By specifically targeting NOX4, it may be possible to intervene in the disease process and alleviate the symptoms associated with asthma. This novel idea holds promise for the future management of asthma, as current treatment options often focus on symptomatic relief rather than addressing the underlying mechanisms driving the disease.

Conclusion

In summary, the findings presented in this manuscript underscore the significance of NOX4 in asthma and provide compelling evidence for its potential as a therapeutic target. Further investigations are warranted to explore the precise molecular mechanisms underlying NOX4-mediated airway remodeling and inflammation, as well as to assess the safety and efficacy of targeting NOX4 in clinical settings. Ultimately, these efforts may lead to the development of innovative and more effective treatment strategies for individuals suffering from asthma.

Ethical Compliance

The animal study was approved by the Ethics Committee of the first affiliated hospital of Bengbu Medical College.

Conflict of Interests

The authors declared no conflict of interest.

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Data Availability Statement

The datasets in this study are available from the corresponding author upon reasonable request.

References