Peroxytinirite augments fibroblast-mediated tissue remodeling via myofibroblast differentiation

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B-TGF-NF-/H9252/H9260 differentiation to myofibroblasts and excessive ECM production via a anti-TGF-B antibody. CAPE completely inhibited the peroxynitrite-augmented MG132 and caffeic acid phenethyl ester (CAPE), and anti-TGF-B inhibitors on the mechanism in this differentiation, the effect of anti-TGF-B inhibitors, and NF-B inhibitors, and the expressions of -smooth muscle actin ( -SMA) and desmin, markers of myofibroblast differentiation, were evaluated. The releases of transforming growth factor- (TGF-), and ECM proteins including fibronectin and collagen I were assessed. To clarify the mechanism in this differentiation, the effect of anti-TGF-B antibody or NF-kB inhibitors on the -SMA expression and ECM production was assessed. Peroxytinirite and SIN-1 significantly augmented the -SMA expression compared with control in a concentration-dependent manner ( and respectively). Peroxytinirite significantly increased desmin and TGF- production ( ). Peroxytinirite enhanced the translocation of NF-kB into the nucleus confirmed by immunocytoasting and immunoblotting. Peroxytinirite, desmin, and TGF- production were blocked by NF-kB inhibitors, MG132 and caffeic acid phenethyl ester (CAPE), and anti-TGF-B antibody. CAPE completely inhibited the peroxytinirite-augmented TGF-B release. The production of fibronectin and collagen I was significantly increased by peroxytinirite ( ) and inhibited by anti-TGF-B antibody. These results suggest that RNS can affect the differentiation to myofibroblasts and excessive ECM production via a NF-kB-TGF-B-dependent pathway.

reactive nitrogen species; airway remodeling; asthma; -smooth muscle actin; nuclear factor-kB

ASTHMA IS A DISORDER CHARACTERIZED by chronic inflammation of the airways, airflow limitation, airway hyperresponsiveness (AHR), and changes in the airway architecture sometimes termed airway remodeling (9). Airway remodeling plays a pivotal role in increasing the severity and irreversible airflow limitation in asthma and leads to the refractoriness of this disease in spite of treatment with high doses of corticosteroids (1a, 8). The parenchymal cells of the airway, including epithelial cells, airway smooth muscles, endothelial cells, and fibroblasts are responsible for the maintenance of the airway structure and are involved in the progression of airway remodeling (27). In airway remodeling, subepithelial fibrosis is one of the pathological features and is characterized by excessive deposition of ECM protein including collagens I, III, and V, fibronectin, and tenasin in the lamina reticularis of the basement membrane (12). In an in vitro model, epithelial injury reportedly differentiated lung fibroblasts into myofibroblasts, which leads to excessive ECM production (24). In a chronic allergen-challenged murine model, the differentiation of fibroblasts to myofibroblasts in the airway was observed (33). In addition, there were more myofibroblasts within the airway walls of asthmatic patients (10, 30), especially refractory asthmatic patients, compared with healthy subjects in pathological examinations suggesting that the differentiation of fibroblasts to myofibroblasts is a key process of airway remodeling in asthma (14).

Excessive production of nitric oxide (NO) during inflammatory and immune processes leads to the formation of reactive nitrogen species (RNS) including peroxynitrite and nitrogen dioxide (4). These RNS are formed from NO and superoxide anion or via the hydrogen peroxide/peroxidase-dependent nitrite oxidation pathway (35). Especially peroxynitrite is an extremely powerful oxidant and is presumed to be largely responsible for many of the adverse effects of excessive NO generation. Excessive RNS production has been reported to cause tissue injury, lipid peroxidation, and nitration of tyrosine residues (35). Furthermore, peroxynitrite caused airway inflammation (31) and AHR (28) in asthmatic animal models. In fact, excessive production of 3-nitrotyrosine, which is a footprint of RNS production, has been observed in the airways of asthmatic patients (15, 29). These data suggest that increased RNS production can be an important contributor to the pathophysiology of asthma.

Transforming growth factor- (TGF-), is a key mediator in a variety of pathological processes, including fibroblast-mediated repair responses. TGF- is believed to be a major regulator of tissue remodeling (5) and is reportedly overexpressed in asthmatic airways (23, 36). It has been reported that the expression of TGF- is modulated by transcriptional factor complexes including NF-kB (7, 18, 19) and activator protein-1 (AP-1) (11, 19). These pathways potentially play a central role in the progression of airway remodeling in asthmatic airways.
The effect of RNS on airway remodeling process has not been fully understood. It has been reported that RNS augment fibroblast-mediated collagen gel contraction and fibroblast chemotaxis toward fibronectin (34), suggesting that RNS can affect the tissue repair process. However, the contribution of RNS to myofibroblast differentiation, which plays a pivotal role in airway remodeling observed in asthma, has not been elucidated yet. In addition, the molecular mechanism involved in the RNS-mediated tissue remodeling process also remains unclear.

The present study was therefore designed first to determine whether RNS could affect the differentiation of lung fibroblasts to myofibroblasts. Next, we assessed the effect of peroxynitrite on the release of TGF-β1 and ECM proteins including fibronectin and collagen I in lung fibroblasts. Finally, we investigated the mechanism of differentiation of lung fibroblasts to myofibroblasts by peroxynitrite.

MATERIALS AND METHODS

Materials. Commercially available reagents were obtained as follows: anti-TGF-β1 antibody (clone: 9016.2), TGF-β1, biotinylated anti-TGF-β1, neutralizing anti-TGF-β antibody, and anti-IgG were from R&D Systems (Minneapolis, MN); peroxynitrite was from...
Upstate Biotechnology (Temescu, CA); 3-morpholinosydnonimine hydrochloride (SIN-1), 3,3',5,5'-tetramethylbenzidine (TMB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), monoclonal anti-human fibronectin antibody, polyclonal anti-human fibronectin antibody, anti-rabbit IgG antibody, and ebselen, a peroxynitrite scavenger, were from Sigma (St. Louis, MO); MG132, a proteasomal inhibitor, and caffeic acid phenethyl ester (CAPE), a specific NF-κB inhibitor (25), were from Calbiochem (La Jolla, CA); DMEM, FCS, and antibiotic-antimycotic were purchased from Invitrogen Life Technologies (Grand Island, NY).

Cell culture. Human fetal lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (Rockville, MD). Normal human adult lung fibroblasts (NHLF) were obtained from Cambrex (Walkersville, MD). The cells were cultured on tissue culture dishes (Falcon; Becton-Dickinson, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin, and 2.5 μg/ml fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. HFL-1 and NHLF cells were passaged every 4–5 days at a 1:4 ratio. HLF-1 cells were used between the 4th and 18th passages and NHLF cells were used between the 4th and 6th passages. To evaluate mediator production in the monolayer culture, cells were seeded in six-well tissue culture plates at a cell density of 1 × 10⁶ per milliliter. At 90% confluence, cells were treated with various concentrations of peroxynitrite in serum-free DMEM (SF-DMEM). For the investigation of the effect of neutralizing anti-TGF-β antibody on peroxynitrite-modulated mediator release, neutralizing anti-TGF-β antibody (10 μg/ml) was also added to the media. The supernatants were harvested after 48 h treatment with peroxynitrite and stored at −80°C until later assay.

Determination of cell viability. For monitoring cell viability, peroxynitrite or vehicle-treated cells were incubated with MTT solution at a final concentration of 1 mg/ml for 4 h at 37°C. After incubation, DMSO was added to each well. The absorbance of each sample at 450 nm with a microreader. Horseradish peroxidase (HRP)-streptavidin (1:20,000 dilution) was added for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H₂SO₄, and the product was quantified at 450 nm with a microreader. Fibronectin was assayed with an ELISA that specifically detects human but not bovine fibronectin (34). Plates were coated with monoclonal anti-fibronectin antibody at 4°C overnight. After being washed three times, standards and samples were added and incubated at room temperature for 2 h. Bound antibody was detected after adding polyclonal anti-human fibronectin antibody (1:2,000 dilution) at room temperature for 1 h. HRP-conjugated anti-rabbit IgG antibody (1:10,000 dilution) was added at room temperature for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H₂SO₄, and the product was quantified at 450 nm with a microreader.

Immunohistochemical localization of α-SMA and NF-κB p65. HFL-1 cells were seeded in an 8-well chamber slide at a density of 1 × 10⁵ per milliliter and cultured for 24 h, and then the medium was replaced with SF-DMEM for 24 h. The cells were incubated with 5 × 10⁻⁵ M peroxynitrite for various time points for NF-κB assessment and for 48 h for α-SMA assessment. After washing, cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100.

Measurement of TGF-β1 and fibronectin. TGF-β1 and fibronectin in the media of the monolayer culture were determined by ELISA (34). Quantification of TGF-β1 was performed as follows: plates were coated with monoclonal anti-TGF-β1 antibody at 4°C overnight. After being washed three times (5 min each), standards and samples were added and incubated at room temperature for 2 h. To measure TGF-β1, all samples were assayed both with and without acidification and neutralization to convert the latent form of TGF-β1 to the active form. To accomplish this, a 500-μl sample was mixed with 100 μl of 1 N HCl and, after 10 min at room temperature, neutralized with 100 μl of 1.2 N NaOH/0.5 M HEPES. Bound antigen was detected after adding biotinylated anti-TGF-β1 antibody for 1 h at room temperature. Horseradish peroxidase (HRP)-streptavidin (1:20,000 dilution) was added for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H₂SO₄, and the product was quantified at 450 nm with a microreader. Fibronectin was assayed with an ELISA that specifically detects human but not bovine fibronectin (34). Plates were coated with monoclonal anti-fibronectin antibody at 4°C overnight. After being washed three times, standards and samples were added and incubated at room temperature for 2 h. Bound antibody was detected after adding polyclonal anti-human fibronectin antibody (1:2,000 dilution) at room temperature for 1 h. HRP-conjugated anti-rabbit IgG antibody (1:10,000 dilution) was added at room temperature for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H₂SO₄, and the product was quantified at 450 nm with a microreader.
X-100 in PBS for 10 min at room temperature and blocked with 1% skim milk in PBS for 1 h at room temperature and rinsed with PBS. Then, they were incubated with mouse monoclonal anti-α/-H9251-SMA antibody (1:400 dilution; Sigma) or mouse monoclonal anti-NF/-H9260p65 antibody (1:100 dilution; Santa Cruz Biotechnology) in 1% skim milk at 4°C overnight. After washing, the cells were incubated with FITC-conjugated anti-mouse IgG antibody (1:1,000 dilution; Sigma) in 1% skim milk for 60 min at room temperature and then viewed with an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) and photographed with a digital camera (DMX1200C; Nikon) under ×400 magnification.

Statistical analysis. Data were expressed as means ± SE. Experiments with multiple comparisons were evaluated by one-way analysis of variance followed by Scheffé test to adjust for multiple comparisons. An unpaired two-tailed Student’s t-test was used for single comparisons. Probability values of <0.05 were considered significant.

RESULTS

To determine whether peroxynitrite induces the differentiation of fibroblasts to myofibroblasts, we assessed the expression of α-SMA, most commonly used as a molecular marker of myofibroblasts, in HFL-1 cells by Western blotting treated with various concentrations of peroxynitrite. Authentic peroxynitrite significantly augmented α-SMA expression compared with the control in a time-dependent manner (at 48 h, 2.6-fold increase; P < 0.01; Fig. 1, A and B). Peroxynitrite also significantly augmented α-SMA expression at 48 h in a concentration-dependent manner (at 10⁻⁵ M, 2.4-fold increase; P < 0.01; Fig. 1, C and D). Furthermore, peroxynitrite induced morphological changes in HFL-1 cells. More stress fibers, estimated for α-SMA immunostaining, were observed in the peroxynitrite-treated cells (Fig. 1E). We also confirmed the peroxynitrite-mediated myofibroblast differentiation by assessing the expression of desmin, which is reportedly another marker of the differentiation (37). The expression of desmin was significantly upregulated by peroxynitrite in a concentration-dependent manner (at 10⁻⁵ M, 1.8-fold increase; P < 0.01; Fig. 1, F and G). The peroxynitrite donor SIN-1 also significantly augmented α-SMA expression in a concentration-dependent manner (at 10⁻⁵ M, 2.1-fold increase; P < 0.05; Fig. 2).

To confirm whether the increased α-SMA expression was directly mediated by peroxynitrite, we assessed the effect of ebselen, a peroxynitrite scavenger, on the peroxynitrite-augmented α-SMA expression. Ebselen completely inhibited the augmentation (at 5 μM; P < 0.01; Supplemental Fig. 1, A and B, available in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site). Furthermore, we investigated the effect of peroxynitrite on cell viability and

Fig. 3. Effect of peroxynitrite on transforming growth factor-β1 (TGF-β1) release by HFL-1. Cultured cells were treated with various concentrations of peroxynitrite. After 48 h, media were harvested and assayed for total or active TGF-β1 by ELISA. All values are expressed as means ± SE for 4 (A; total TGF-β1) or 3 (B; active TGF-β1) separate experiments. *P < 0.05, **P < 0.01, compared with the values of control.

Fig. 4. Effect of peroxynitrite on α-SMA expression and TGF-β1 release in normal human adult lung fibroblasts (NHLF). NHLF cells were treated with various concentrations of peroxynitrite for 48 h. Cells and media were harvested. α-SMA expression was analyzed by Western blotting (A) and quantified by densitometry (B). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. Media were assayed for total TGF-β1 by ELISA (C). All values are expressed as means ± SE for 3 separate experiments. *P < 0.05, **P < 0.01, compared with the values of control.
proliferation because high doses of peroxynitrite have been reported to have cytotoxic effect. A concentration of $10^{-5}$ M or less peroxynitrite has no cytotoxic effects, and cell proliferation was not affected by $10^{-5}$ M peroxynitrite (Supplemental Fig. 1, C and D).

To determine whether peroxynitrite augments TGF-β release by HFL-1 cells, we measured the TGF-β concentration in the media. Both total and active TGF-β amounts were accumulated in the media in a time-dependent manner (data not shown). Peroxynitrite significantly increased total TGF-β (at $10^{-5}$ M, $587 \pm 69$ vs. $251 \pm 44$ pg/ml; $P < 0.01$; Fig. 3A) and active TGF-β release (at $10^{-5}$ M, $51.0 \pm 1.7$ vs. $26.3 \pm 0.9$ pg/ml; $P < 0.01$; Fig. 3B) from HFL-1 cells in a concentration-dependent manner.

We assessed the effect of peroxynitrite on α-SMA expression and TGF-β release in NHLF. Peroxynitrite significantly augmented α-SMA expression (at $10^{-5}$ M, 3.5-fold increase; $P < 0.01$; Fig. 4, A and B) and total TGF-β release (at $10^{-5}$ M, $184 \pm 25$ vs. $52 \pm 28$ pg/ml; $P < 0.05$; Fig. 4C).

To clarify how peroxynitrite augments TGF-β release by HFL-1 cells, we assessed the translocation of NF-κB into the nucleus, which is thought to regulate TGF-β expression. Translocation of NF-κB p65 into the nucleus was assessed by immunocytostaining and Western blotting. After treatment

![Fig. 5. Effect of peroxynitrite on translocation of NF-κB p65 into nucleus in HFL-1. HFL-1 cells were treated with $10^{-5}$ M peroxynitrite for 0, 30, 60, 90, and 120 min, and the intracellular localization of NF-κB p65 was determined by immunocytostaining (A). The amount of NF-κB p65 in the nuclear fraction was analyzed by Western blotting (B) and quantified by densitometry (C). Each NF-κB p65 band intensity was normalized with the corresponding lamin A/C band intensity. All values are expressed as means ± SE for 4 separate experiments. **$P < 0.01$, compared with the values of control. Bars = 50 μm.](http://ajplung.physiology.org/)
with 10^{-5} M peroxynitrite, the translocation of NF-κB p65 was clearly enhanced at 60 min, and the fluorescence intensity of NF-κB p65 in the nucleus was diminished at 120 min (Fig. 5A). The enhanced nuclear translocation of NF-κB p65 was also confirmed by Western blotting. The amount of NF-κB p65 in the nucleus was significantly increased at 60 min compared with the control (2.7-fold increase; \( P < 0.01 \); Fig. 5, B and C) and decreased at 120 min (Fig. 5, B and C). To investigate whether NF-κB is related to α-SMA expression in HFL-1 cells, the effects of MG132, a proteasomal inhibitor and CAPE, a specific NF-κB inhibitor, on the α-SMA expression were evaluated. The peroxynitrite-augmented α-SMA expression was completely inhibited by both inhibitors (Fig. 6, A–D).

Furthermore, CAPE completely inhibited the peroxynitrite-augmented TGF-β1 release (at 3 μg/ml; 315 ± 28 vs. 99 ± 21 pg/ml; \( P < 0.01 \); Fig. 6E).

To clarify the mechanistic role of TGF-β1 in peroxynitrite-mediated α-SMA expression in HFL-1 cells, we investigated the effect of neutralizing anti-TGF-β antibody on α-SMA expression. Neutralizing anti-TGF-β antibodies significantly reduced the peroxynitrite-augmented α-SMA expression compared with the control IgG-treated group (at 10^{-5} M, 2.4-fold increase vs. 1.3-fold increase; \( P < 0.01 \); Fig. 7, A and B).

To determine whether peroxynitrite stimulates the production of ECM proteins, the release of fibronectin and expression of collagen I were assessed by ELISA and Western blotting, respectively. Peroxynitrite significantly augmented the fibronectin (at 10^{-5} M, 2,178 ± 176 vs. 698 ± 41 ng/ml; \( P < 0.01 \); Fig. 8A) and collagen I production (at 10^{-5} M, 2.0-fold increase; \( P < 0.01 \); Fig. 8, B and C) in a concentration-dependent manner. Neutralizing anti-TGF-β antibodies significantly inhibited the peroxynitrite-augmented production of fibronectin (at 10^{-5} M, 1,958 ± 65 vs. 816 ± 49 ng/ml; \( P < 0.01 \); Fig. 8A).
DISCUSSION

The present study demonstrated that peroxynitrite significantly augmented α-SMA expression in HFL-1 and NHLF cells. Peroxynitrite enhanced TGF-β1 release by HFL-1 and NHLF cells and also promoted the translocation of NF-κB into the nucleus. A proteasomal inhibitor, MG132, and a specific NF-κB inhibitor, CAPE, completely inhibited the peroxynitrite-augmented α-SMA expression. In addition, CAPE diminished the peroxynitrite-augmented TGF-β1 release. Neutralization of TGF-β significantly inhibited the peroxynitrite-augmented α-SMA expression. Furthermore, fibronectin and collagen I production were significantly enhanced by peroxynitrite, which was inhibited by neutralizing anti-TGF-β antibody. These data suggest that RNS can induce the differentiation of lung fibroblasts to myofibroblasts and the excessive production of ECM protein via a NF-κB-TGF-β1-dependent pathway.

It has been reported that the differentiation of lung fibroblasts to myofibroblasts plays a pivotal role in the development of airway remodeling in asthma (14). In fact, Schmidt et al. (30) also described that the accumulation of circulating fibrocytes was observed in the airways of asthmatic patients and that these were precursors of bronchial myofibroblasts. Brewster et al. (6) showed that more myofibroblasts were observed in the subepithelial basement membrane-collagen layer of asthmatic airways compared with healthy subjects. In the current study, we clearly showed that peroxynitrite, which is one of the RNS and is an excessively produced NO-related molecule in the airways of asthmatic patients, augmented the differentiation of lung fibroblasts to myofibroblasts.

TGF-β1 is reported to be a key mediator of airway remodeling (17, 22), and the gene expression of TGF-β1 is reportedly regulated by NF-κB (7, 18, 19) and AP-1 (11, 19). Broide et al.

![Graph](AJP-Lung Cell Mol Physiol • VOL 295 • NOVEMBER 2008 • www.ajplung.org)
TGF-β1 had similar effects on the protein production in fetal fibroblasts challenged mice, whereas the TGF-β1 level in IκB kinase knockout mice was decreased compared with wild-type mice, and the airway remodeling was ameliorated. These findings suggest that NF-κB plays a key role in the regulation of TGF-β1 gene expression. In a variety of cells, peroxynitrite is reported to enhance NF-κB DNA binding activity (2, 3, 16, 21). In the current study, peroxynitrite promoted the translocation of NF-κB p65 into the nucleus in HFL-1 cells and stimulated TGF-β1 release, which was inhibited by a NF-κB inhibitor. Taken together, peroxynitrite could augment TGF-β1 release by lung fibroblasts through NF-κB activation.

The possible mechanism for the activation of NF-κB by peroxynitrite is as follows: according to a previous study, peroxynitrite nitrates tyrosine 181 residues of IκBα, which consequently leads to the dissociation of intact IκBα from NF-κB (38). Peroxynitrite itself has also been reported to activate NF-κB without preactivation by proinflammatory mediators such as TNF-α and LPS (2, 3, 16, 21). However, the regulation of NF-κB by nitrative stress has not been fully elucidated. Therefore, further study is needed.

Excessive deposition of ECM proteins crucially contributes to airway remodeling. It has been reported that myofibroblasts can produce greater amounts of ECM proteins compared with undifferentiated fibroblasts (13). However, the precise effect of RNS on ECM production in HFL-1 cells remains unknown. TGF-β reportedly induces myofibroblast differentiation and subsequent ECM protein synthesis (20). The current study demonstrated that peroxynitrite augmented the α-SMA expression and ECM production by HFL-1 cells. This augmentation was inhibited by neutralizing anti-TGF-β antibody, and our data support the previous study (1). Taken together, RNS can enhance the myofibroblast differentiation and ECM production via a TGF-β1-dependent pathway.

Although peroxynitrite has a very short half-life (∼1.5 s), TGF-β1 was accumulated in the media in a time-dependent manner (data not shown). The possible reason is as follows: the gene expression of TGF-β1 was triggered after NF-κB activation, and subsequently the produced TGF-β1 induced myofibroblast differentiation. Differentiated myofibroblasts can produce greater amounts of TGF-β1, and this positive feedback would be related to TGF-β1 accumulation in the media.

Because fetal lung fibroblasts may not respond to peroxynitrite the same way as postnatal lung fibroblasts, we investigated the effects of peroxynitrite on the α-SMA expression and TGF-β1 release in NHLF. As shown in Fig. 4, peroxynitrite had similar effects on the protein production in fetal fibroblasts and adult fibroblasts. These results are compatible with those of our previous study (34).

High doses of peroxynitrite have cytotoxic effects. In this study, we used noncytotoxic doses of peroxynitrite (∼10−5 M) as shown in Supplemental Fig. 1C. A concentration of 10−5 M peroxynitrite has no proliferative effect (Supplemental Fig. 1D). Furthermore, ebselen, a peroxynitrite scavenger, diminished the peroxynitrite-mediated profibrotic response. These data suggest that peroxynitrite directly altered lung fibroblasts to the profibrotic phenotype.

Peroxynitrite can enhance the production of IL-8 and TNF-α via NF-κB activation (16, 21). Recently, we showed that 3-nitrotyrosine, a footprint of RNS production, was excessively expressed in the sputum cells from refractory asthmatic patients compared with well-controlled asthmatic patients (32). In addition, the amount of 3-nitrotyrosine was well-correlated with the degree of airflow limitation in the asthmatic patients. Although the possible mechanisms by which RNS are related to the pathogenesis of refractory asthma are still unclear, peroxynitrite-mediated tissue remodeling may be involved in the refractoriness of asthma.

In conclusion, the current study shows that RNS can enhance NF-κB activation and TGF-β1 release in HFL-1 cells, and consequently myofibroblast differentiation and excessive ECM production are induced. Therefore, the NF-κB-TGF-β1 pathway is thought to play a pivotal role in the differentiation of lung fibroblasts to myofibroblasts. Our data suggest that the modulation of this pathway may have therapeutic potential for airway remodeling.

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