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RESEARCH ARTICLE

NADPH and xanthine oxidases control induction of inflammatory mediator expression by organic dust in the lung

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Dedicated to the memory of Usha R. Pendurthi, Ph. D., a great friend and colleague who has made outstanding contributions in the field of thrombosis and hemostasis.

Abstract

Exposure to organic dust in animal and agricultural farms and the ensuing lung inflammation are linked to the development of respiratory diseases. We found previously that elevated production of reactive oxygen species (ROS) by aqueous poultry organic dust extract (hereafter referred to as dust extract) mediates induction of proinflammatory mediators in airway epithelial cells. In the present study, we investigated whether ROS generated by NADPH oxidases (NOX) and xanthine oxidase (XO) controls induction of inflammatory mediators by dust extract and the underlying mechanisms in bronchial epithelial cells. Using chemical inhibitors and siRNA targeted knockdown, we found that NOX1, NOX2, NOX4, and XO-derived ROS regulates induction of proinflammatory mediator levels. Like airway epithelial cells in vitro, NOX inhibitor VAS2870 reduced keratinocyte chemoattractant (KC), IL-6, and TNF-α production and 4-hydroxynonenal (4-HNE) staining induced by dust extract in mouse lungs. VAS2870 inhibition of proinflammatory mediators was associated with reduced NFκB and Stat3 activation indicating that NOX generated ROS activates NFκB and Stat3 to induce proinflammatory gene expression. Dust extract increased the membrane association of $p47^{phox}$ in airway epithelial cells indicating NOX2 activation but had no effect on NOX2 protein levels. In summary, our studies have shown that NOX and XO generated ROS control organic dust induction of proinflammatory mediators in airway epithelial cells via NFκB and Stat3 activation.

KEYWORDS

cytokines, gene regulation, occupational lung diseases, oxidative stress

Abbreviations: 4-HNE, 4-hydroxynonenal; BEGM, bronchial epithelial growth medium; CDDOIm, 1-(2-cyano-3,12,28-trioxooleana-1,9(11)-dien-28-yi)-1H-imidazole; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium; DUOX, dual oxidase; KC, keratinocyte chemoattractant; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPK, mitogen activated protein kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NHBE, normal human bronchial epithelial; NOX, NADPH oxidase; PK, protein kinase; ROS, reactive oxygen species.

1 | INTRODUCTION

Occupational lung diseases are the major cause of work-related illness in the United States.[1] This is particularly true for workers engaged in livestock and crop farming as they spend prolonged periods of time in their work environments that are contaminated with high concentrations of airborne dust.[2] Exposure to animal farm dust or grain dust is correlated with greater prevalence of respiratory symptoms and respiratory diseases such as asthma, chronic bronchitis, organic dust toxic syndrome, and hypersensitivity pneumonitis.[2] In fact, epidemiological studies have indicated that farmers are at greater risk for occupational asthma than workers in non-agricultural occupations.[3] In particular, poultry farm workers are reported to experience higher prevalence and greater severity of respiratory symptoms and respiratory diseases than workers in swine and cattle farms.[4] Workers in poultry farms are exposed to higher concentrations of organic dust and its constituents that may explain the higher prevalence and greater severity of respiratory symptoms and respiratory diseases.[4]

Organic dust is a complex mixture of organic and inorganic materials containing food particles, fecal material, dander, feathers, mites, fungi, bacteria, viruses, and toxic gases.[5,6] Animal farm dusts tend to contain higher concentrations of microbes and microbial components such as endotoxin, peptidoglycan, and fungal toxins.[7] Recently, we reported that poultry organic dust contains bacterial extracellular vesicles that induce proinflammatory mediators in airway epithelial cells and in mouse lungs to cause neutrophilic inflammation.[8] Exposure of lung cells in vitro[9–12] and experimental mice[13–15] to aqueous organic dust extracts and exposure of human subjects[16,17] to animal farm environment causes increased production of cytokines, chemokines, and other inflammatory proteins. Repetitive intranasal instillation of aqueous organic dust extract into mice produced lung histological changes including peribronchial infiltration of inflammatory cells and increased collagen staining around airways.[14,15] We previously reported that aqueous poultry organic dust extracts increased IL-8 expression in lung epithelial and THP-1 monocytic cells by increasing gene transcription via activation of nuclear factor κB (NFκB) and activator protein-1 (AP-1) binding and protein kinase C (PKC) and mitogen activated protein kinase (MAPK) signaling pathways.[9] Treatment of lung epithelial and THP-1 cells with poultry organic dust extracts increased the expression of cytokines, chemokines, intercellular adhesion molecule-1 (ICAM-1), toll-like receptor (TLR)-4, prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 (PTGS2/COX2), superoxide dismutase 2 (SOD2) and transcription factors such as early growth response 1 (EGR1), Jun, Fos B and activating transcription factor 3 (ATF3) indicating effects on cellular networks controlling immune and inflammatory responses.[18] Trypsin- and elastase-like protease activities present in poultry organic dust extracts and increased reactive oxygen species (ROS) production acting via PKC and NFκB activation were found to control induction of inflammatory mediator expression.[19] Because NADPH oxidase (NOX) and xanthine oxidase (XO) enzymes are major producers of cellular ROS in inflammatory diseases,[20,21] we hypothesized that ROS overproduction by NOX and XO enzymes contributes to the induction of inflammatory mediators by poultry organic dust extracts in lung epithelial cells. The importance of NOX and XO enzymes in the control of organic dust induction of lung inflammation has not been reported previously. Some of the data reported here have been presented in the form of a conference abstract.[22]

NOX are a family of multi-subunit enzymes, majority of which are localized on the plasma membrane, whose primary function is production of superoxide anion (O_2^-) and/or hydrogen peroxide (H_2O_2).[23] NOX1, NOX2, NOX3, and NOX5 generate O_2^-, whereas NOX4 and dual oxidase (DUOX)-1 and -2 generate H_2O_2. It is not entirely clear if NOX4, DUOX1, and DUOX2 primarily produce H_2O_2; it has been speculated that their primary product is O_2^- which rapidly undergoes dismutation preventing its detection.[24,25] NOX enzymes are expressed by a wide variety of cells including endothelial, epithelial, smooth muscle, fibroblast, and immune cells. NOX enzymes are localized in plasma membrane except for NOX4 which has been reported to be present in the mitochondria and the nucleus. Published studies have shown that lung epithelial cells express NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2.[26] In agreement with these studies our preliminary studies found that Beas2B bronchial and primary normal human bronchial epithelial (NHBE) cells express mRNAs for all the NOX enzymes (unpublished). XO which catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid to produce ROS has been implicated in the development of respiratory and cardiovascular diseases.[27] Herein, we studied the contributions and the mechanisms by which NOX and XO enzymes and other ROS producers such as mitochondria control dust extract induction of inflammatory mediator expression in airway epithelial cells in vitro and in mouse lungs.

2 | MATERIALS AND METHODS

2.1 | Chemicals and antibodies

Diphenyleneiodonium (DPI) (Sigma-Aldrich), VAS2870 (Enzo Life Sciences), ML171 (Tocris Bioscience) and GKT137831 (Cayman Chemical), and Febuxostat

(Sigma-Aldrich) were dissolved in dimethylsulfoxide (DMSO). MitoTEMPO hydrate (Cayman Chemical) was dissolved directly in cell culture medium. 2′7′-dichlorodihydrofluorescein diacetate (DCFDA) and MitoSOX Red (Invitrogen) were dissolved in DMSO. Cremophor (Kolliphor EL) was from Sigma-Aldrich. siRNAs targeting NOX1, NOX2 and NOX4 were purchased from Santa Cruz Biotechnology or from Sigma-Aldrich. IL1β (1:1000, 12703, Cell Signaling Technology), COX-2 (PTGS2), (1:1000, A303-600A, Bethyl Laboratories), ICAM-1 (1:1000, sc-8439, Santa Cruz Biotechnology), NOX2 (gp91) (1:1000, sc-130543, Santa Cruz Biotechnology), p47phox (1:100, SAB4502809, Millipore Sigma, for immunofluorescence staining), phospho-NF-κB p65 (S536) (1:1000, 3033, Cell Signaling), phospho-Stat3 (1:1000, 9145, Cell Signaling) and Stat3 (1:1000, 4904, Cell Signaling Technology), NF-κB p65 (1:1000, sc-372, Santa Cruz Biotechnology), and β-actin (1:1000, sc-47778, Santa Cruz Biotechnology) antibodies were used as primary antibodies in western blotting analysis. AP-linked anti-mouse (1:5000, 7056), AP-linked anti-rabbit (1:5000, 7054), HRP-linked anti-rabbit (1;1000, 7074), and HRP-linked anti-mouse (1:5000, 7076) from Cell Signaling Technology were used as secondary antibodies in western blotting analysis. 4-HNE antibodies (1:100, ab46545, Abcam) were used for immunohistochemical analysis of 4-HNE levels in mouse lung sections.

2.2 | Dust extract preparation

Settled dust (organic dust) collected on vertical surfaces was obtained from two different commercial poultry broiler farms located in East Texas when the chickens were 7–8 weeks of age and stored at −70°C. Aqueous extract of organic dust was prepared using Kaighn's modification of Ham's F-12 medium (F-12K medium) or endotoxin-free Dulbecco's phosphate-buffered saline (DPBS) at a ratio of 1:10 (w/v) as described previously.[9] The resulting dust extract was considered arbitrarily as 100%. Our studies have shown that dust extracts prepared from several dust samples from two different poultry farms in East Texas produced similar inductive effects on inflammatory mediators, ROS production, and NFκB and Stat3 activation in bronchial epithelial cells[8,9,19,28] indicating the reproducibility effects of dust extract samples.

2.3 | Cell culture

Beas2B bronchial epithelial cells (ATCC TIB-202) were grown on plastic culture dishes coated with fibronectin, bovine type I collagen, and bovine serum albumin in LHC-9 medium (Invitrogen) and NHBE cells from male and female donors (Lonza or Lifeline Cell Technology) were grown in bronchial epithelial growth medium (BEGM) (Lonza) or bronchia life complete medium (Lifeline Cell Technology). When cells were approximately 80% confluent, they were maintained in RPMI 1640 medium without serum overnight and subjected to treatments in the same medium.

2.4 | Cell viability

Viabilities of Beas2B and NHBE cells were determined by MTS assay (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega).

2.5 | Mice

Animal experiments had been approved by our Institutional Animal Care and Use Committee. Female C57BL6 mice (Jackson Laboratories) of 8−10 weeks of age were quarantined for 1 week prior to treatments. Mice were fed standard diet and water and maintained under 12 h light/dark cycle. Mice were administered NOX inhibitor, VAS2870 (Millipore Sigma) (25 mg/kg, 200 μl) dissolved in a carrier solution containing 80% DPBS, 10% DMSO, and 10% Cremophor (Kolliphor EL) via intraperitoneal injection 1 h prior to treatment with dust extract. Control mice received only the carrier solution. Mice were anesthetized by intraperitoneal injection of ketamine-xylazine (0.1 ml/20 g body weight mice) and 50 μl of 10% dust extract in D-PBS or 50 μl D-PBS was administered intranasally into mice. After 3 h, mice were euthanized, and lungs were obtained for immunohistochemical staining, preparation of homogenate, and RNA extraction.

2.6 | Western blotting

Protein concentrations of cell lysate and lung homogenate were determined by Bradford assay. Equal amounts of protein (15–30 μg) were separated by SDS-PAGE on 10% NuPAGE Bis-Tris gel (Invitrogen) in MOPS running buffer and separated proteins were transferred by electroblotting to PVDF membrane. Membrane was reacted sequentially with primary and secondary antibodies and protein bands were visualized by the enhanced chemifluorescence (ECF) or enhanced chemiluminescence (ECL) detection using Bio-Rad Molecular Imager FX or Bio-Rad Chemidoc MP Imager. Protein bands were quantified using Quantity One or ImageLab software (Bio-Rad). Actin was used as a loading control to normalize the protein levels.

2.7 | ELISA

The levels of IL-6, IL-8, keratinocyte chemoattractant (KC), TNF-α, and IL-1β in cell culture medium and in mouse lung homogenates were determined by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instruction (R & D laboratories).

2.8 | RNA isolation and Real-Time quantitative RT-PCR

Total RNA was isolated using TRI reagent (Molecular Research Center) and RNA purity was checked by determining the 260/280 and 260/230 ratios. cDNA was synthesized using iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad Laboratories). Typically, 1 μg of total RNA was reverse transcribed using oligo (dT) and random primers in a reaction volume of 20 μl. Resulting cDNA was diluted 4× and 2.5 μl of diluted cDNA was used in amplification reactions. For measurement of 18S rRNA, cDNA was diluted 200× and 2.5 μl of diluted cDNA was used in amplification reactions. The levels of mRNAs were determined by TaqMan gene expression assays (Thermo Fisher Scientific) under reaction conditions of 40 cycles of 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s with a CFX 96 Real-Time PCR Detection System (Bio-Rad). mRNA levels were normalized to18S rRNA or actin mRNA levels. Normalized gene expression data (ΔΔCt) relative to control (untreated sample) set to 1 was obtained using CFX Manager Software (Bio-Rad). TaqMan gene expression assay IDs used for the quantification of mRNAs are listed in Table 1.

2.9 | siRNA-mediated knockdown

Scrambled siRNAs and siRNAs targeting human NOX proteins were purchased from Santa Cruz Biotechnology or Sigma-Aldrich. siRNAs were transfected into Beas2B and NHBE cells using Lipofectamine 2000/3000 (Invitrogen) or RNAiMax (Invitrogen) according to the manufacturer's instructions. Incubations were continued for 6 and 24 h for Beas2B and NHBE cells, respectively, and medium replaced with fresh medium and cells grown for 48–72 h, at which time they were washed twice with RPMI medium without serum and subjected to treatments in the same medium.

2.10 | Visualization of intracellular ROS generation in bronchial epithelial cells

Semi-confluent Beas2B and NHBE cells were incubated overnight in RPMI medium without phenol red and serum. Cells were first incubated with NOX chemical inhibitors for 1 h and treated with 1% dust extract for 30 min. Afterwards, cells were incubated with 10 μM dichlorodihydrofluorescein diacetate (DCFDA) for 30 min. To measure mitochondrial ROS generation, cells were first treated with 1% dust extract for 60 min and incubated with 10 μM MitoSOX Red for 30 min. Cells were washed twice with PBS and images captured with ZOE Fluorescent Cell Imager (Bio-Rad). To quantify the fluorescent intensity, intensities of individual cells were measured using ImageJ software (NIH).

2.11 | Immunohistochemical staining

Mice were euthanized by injecting barbiturate euthanasia solution and exsanguinated by transecting renal artery. Lungs were cleared of blood by injecting 10 ml of D-PBS into the right heart ventricle and afterwards instilled with Excell Plus fixative (American MasterTech) under a constant hydrostatic pressure of 20 cm via a canula inserted into the trachea. After the trachea was tied off with suture thread, lungs were stored in the fixative for at least 48 h before processing for sectioning. Sections were

TABLE 1 List of TaqMan gene expression assays used for quantification of mRNA levels

Gene symbol	Gene name	Assay ID
IL-8	Interleukin-8	Hs00174103_m1
ICAM-1	Intercellular adhesion molecule -1	Hs00164932_m1
IL-1β	Interleukin-1β	Hs01555410_m1
IL-6	Interleukin-6	Hs00985639_m1
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase 2/ cyclooxygenase-2)	Hs00153133_m1
NOX1	NADPH oxidase 1	Hs01071082_m1
NOX4	NADPH oxidase 4	Hs01558201_m1
Actin	Actin	Hs01060665_g1
18S	18S ribosomal RNA	Hs99999901_s1

processed for 4-HNE immunohistochemical staining using VECTASTAIN Elite ABC kit (Vector Laboratories) and 3-amino-9-ethylcarbazole (AEC) substrate according to the kit instructions. Immunohistochemical data were blinded.

2.12 | Immunofluorescence staining

Beas2B cells were grown on coverslips coated with fibronectin, bovine type I collagen, and bovine serum albumin in LHC-9 medium and maintained in serum-free RPMI 1640 medium containing antibiotics overnight prior to treatment. After treatment, cells were washed twice with ice-cold PBS and subjected to fixation and permeabilization. Afterwards, cells were sequentially incubated with anti-$p47^{phox}$ antibody and FITC-conjugated secondary antibody. Nuclei were visualized by staining with 4′,6′-diamidino-2-phenylindole (DAPI). Images were captured with a confocal microscope (Zeiss).

2.13 | Statistical analysis

Data are shown as mean ± SE. Statistical significance between the two treatment groups was evaluated by two-tailed unpaired *t*-test and that among three or more groups was evaluated by one-way ANOVA using Tukey's multiple-comparison test.

3 | RESULTS

3.1 | Effects of NOX chemical inhibitors on the induction of inflammatory mediators

We found previously that treatment of lung epithelial cells with dust extract increased the production of ROS.[19,28] Furthermore, we found that antioxidants suppressed induction of inflammatory mediators suggesting that ROS control their induction.[19,28] As NOX enzymes are major producers of ROS, we determined the effects of NOX chemical inhibitors on the induction of proinflammatory mediators to understand the contributions of different NOX enzymes. In our initial studies, the classical NOX inhibitor DPI inhibited proIL-1β, PTGS2, ICAM-1, IL-8, and IL-6 proteins induced by dust extract in Beas2B and NHBE cells (Figure S1) indicating a potential role for NOX enzymes in the induction of inflammatory mediators. As DPI is a non-selective NOX inhibitor and exerts many off-target effects, we determined the effects of other NOX inhibitors with improved selectivity such as VAS2870, ML171, and GKT137831[29–31] on the induction of inflammatory mediators. Treatment with VAS2870 significantly inhibited proIL-1β, PTGS2, ICAM-1, IL-8, and IL-6 protein levels induced by dust extract in Beas2B (Figure 1A–E) and NHBE cells (Figure 2A–D). We could not detect IL-6 in medium of control and dust extract treated NHBE cells consistent with our previously published findings.[28] VAS2870 reduced IL-1β, PTGS2, ICAM-1, and IL-8 but not IL-6 mRNA levels in Beas2B cells treated with dust extract (Figure 1F–J). Although VAS2870 potently inhibits NOX2, it is not a NOX2 isoform-specific inhibitor.[31] Therefore, we investigated the effects of ML171 and GKT137831 which are reported to show selectivity for the inhibition of NOX1 and NOX4,[30] on dust extract induction of inflammatory mediators whereas ML171 at low concentration (0.25 μM) appears to be selective for inhibition of NOX1, GKT137831 is known to inhibit NOX1 and NOX4. Treatment with 1 and 5 μM ML171 did not inhibit any of the inflammatory mediator protein levels analyzed (unpublished) but 10 μM ML171 inhibited ICAM-1 and appeared to inhibit proIL-1β (two-tailed *p*-value = 0.0899) and PTGS2 (two-tailed *p*-value = .0894), but not IL-6 and IL-8 protein levels (Figure 1K–O). We did not determine the effects of ML171 on induction of inflammatory mediators in NHBE cells. On the other hand, treatment with GKT137831 reduced proIL-1β, ICAM-1, and PTGS2 but not IL-6 and IL-8 protein levels in Beas2B cells (Figure 1P–T) and reduced ICAM-1 but not proIL-1β, PTGS2, and IL-8 protein levels in NHBE cells (Figure 2E–H). Treatment with DPI but not VAS2870 reduced the viabilities of Beas2B and NHBE cells (Figure S2). Collectively, these data indicated that NOX enzymes play a role in the regulation of induction of inflammatory mediator expression by dust extract.

3.2 | Effects of RNAi knockdown of NOX enzymes on inflammatory mediator production

The effects of pharmacological inhibitors although indicated that NOX enzymes are important for the dust extract induction of inflammatory mediators, the results may not inform the relative contributions of NOX1, NOX2, and NOX4 as the chemical inhibitors have overlapping inhibitory effects on NOX enzymes. As RNAi mediated knockdown allows target-specific reduction of protein levels, we determined the effects of knockdown of NOX enzymes on the induction of inflammatory mediator protein levels in Beas2B and NHBE cells. Knockdown experiments resulted in 40%–60% reduction in protein or mRNA levels of NOX enzymes. NOX1 and NOX4 antibodies did not produce reliable western blotting results hence we determined their mRNA levels by qRT-PCR. Knockdown of NOX1 in Beas2B cells caused significant reductions in proIL-1β, ICAM-1, IL-8, and IL-6 protein levels (Figure 3A–E). Likewise knockdown of NOX2 levels caused significant

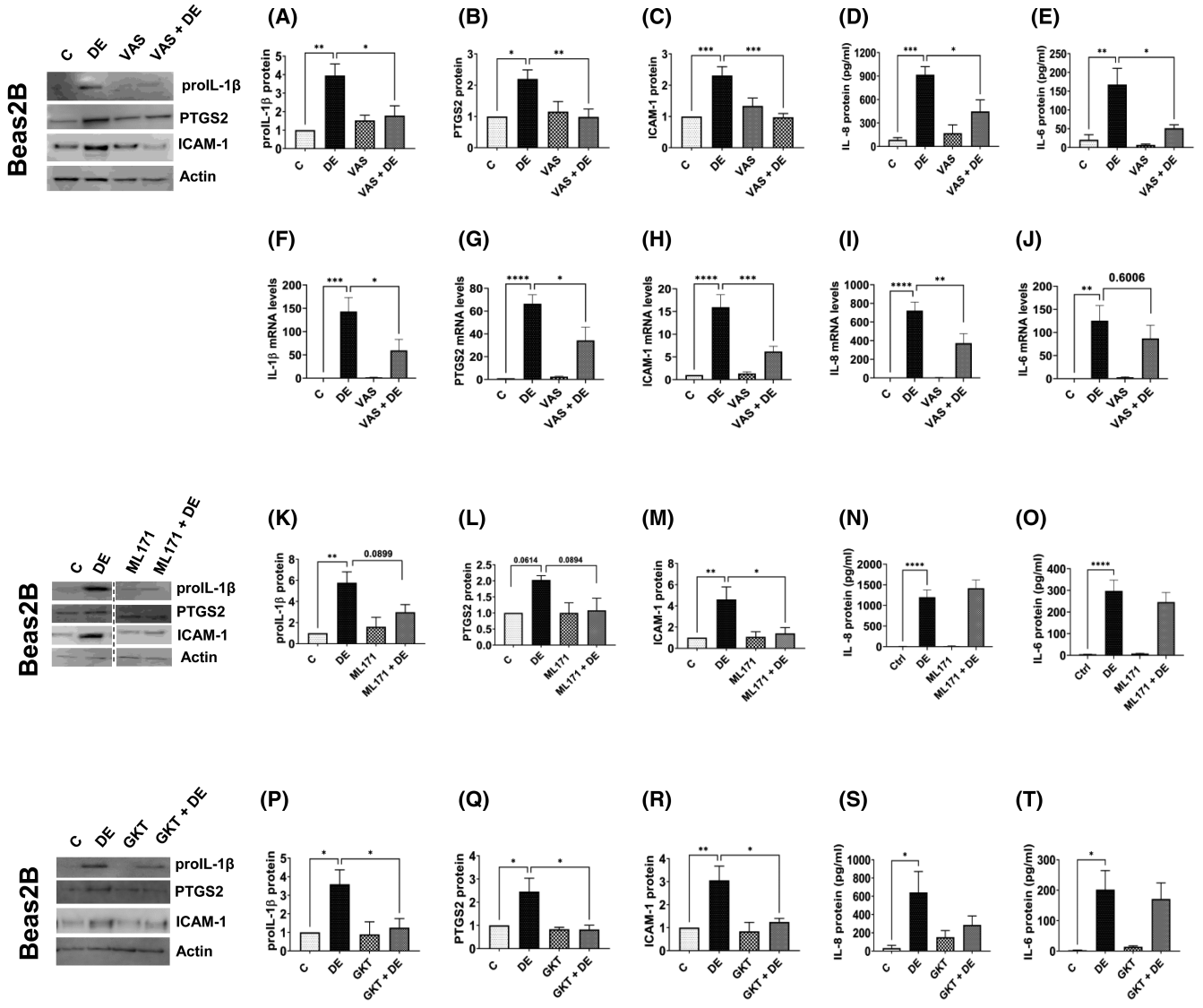

FIGURE 1 Effects of NOX chemical inhibitors on dust extract induction of inflammatory mediators in Beas2B bronchial epithelial cells. Cells were incubated with or without VAS2870 (5 μM) (A–J), ML171 (10 μM) (K–O) and GKT137831 (10 μM) (P–T) for 1 h before treatment with medium (C) or dust extract (DE) (0.25%) for 3 h. Levels of proIL-1β, PTGS2 and ICAM-1 proteins in cell lysates and IL-8 and IL-6 proteins in cell medium and their mRNAs were determined. Cellular protein levels were normalized to actin, and mRNA levels were normalized to 18S rRNA or actin mRNA levels. Representative western blots are shown. Noncontiguous lanes in western blot are indicated by a broken line. Data shown are mean ± SE ($n = 5–6$). *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

suppression of proIL-1β, ICAM-1, IL-8, and IL-6 protein levels in Beas2B cells (Figure 3F–J). Knockdown of NOX4 significantly reduced proIL-1β, ICAM-1, IL-8, and IL-6 protein levels (Figure 3O–S). In NHBE cells, reduction in NOX2 levels resulted in the inhibition proIL-1β and ICAM-1 but not IL-8 levels ($p = .1726$; Figure 3K–N).

3.3 | Effects of NOX inhibitors on ROS production

Our chemical inhibitor and siRNA knockdown studies pointed to NOX1, NOX2, and NOX4 as playing important roles in the dust extract induction of inflammatory mediator expression. Because NOX enzymes are major producers of ROS, we tested if NOX chemical inhibitors suppress dust extract induction of ROS in Beas2B and NHBE cells. We found that dust extract increased DCFDA staining indicating increased ROS production that was suppressed by VAS2870 and GKT137831 (Figure 4A–D) and ML171 (Figure 4G,H). These data together with the data of the effects of NOX chemical inhibitors and NOX knockdown on inflammatory mediator expression (Figures 1–3) suggested that NOX derived ROS mediates induction of inflammatory mediator expression. We further investigated the role of NOX2 by siRNA-targeted knockdown

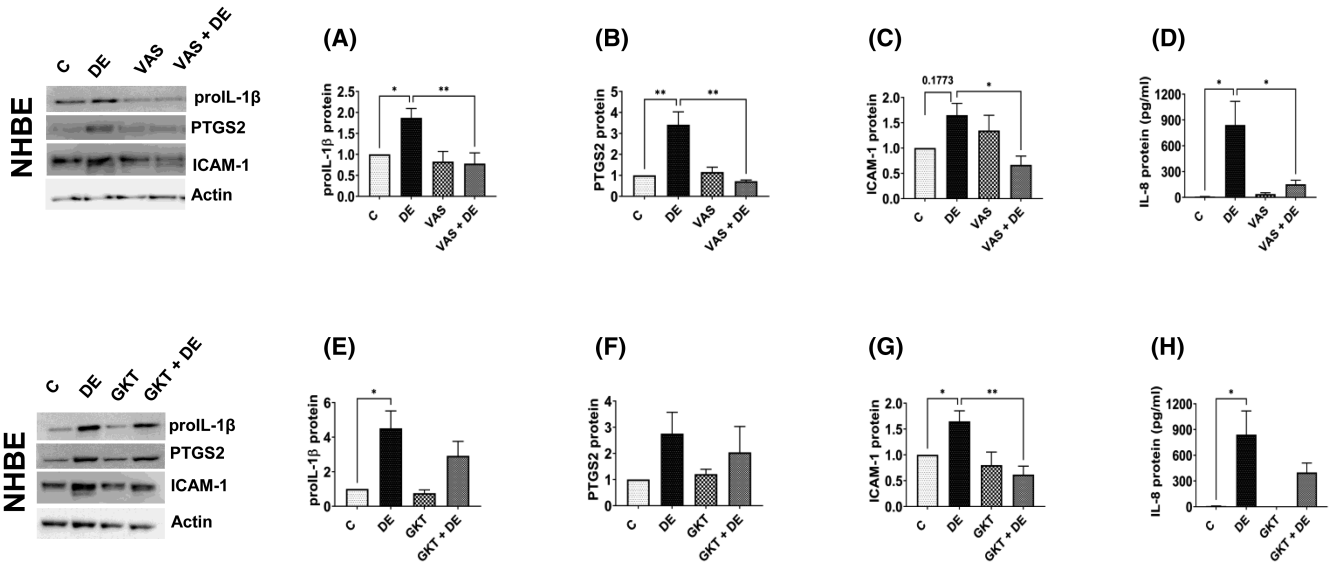

FIGURE 2 Effects of NOX chemical inhibitors on dust extract induction of inflammatory mediators in normal human bronchial epithelial (NHBE) cells. Cells were incubated with or without VAS2870 (5 μM) (A–D) or GKT137831 (10 μM) (E–H) for 1 h before treatment with medium (C) or dust extract (DE) (0.25%) for 3 h. Levels of proIL-1β, PTGS2 and ICAM-1 proteins in cell lysates and IL-8 protein in cell medium were determined. Cellular protein levels were normalized to actin. Representative western blots are shown. Data shown are mean ± SE ($n = 4$). *$p < .05$, **$p < .01$.

experiments. Results demonstrated that knockdown of NOX2 in Beas2B cells suppressed dust extract induction of ROS production consistent with a role for NOX2 in the generation of ROS (Figure 4E,F).

3.4 | Effects of dust extract on mitochondrial ROS levels

Mitochondria are an important source of ROS in mammalian cells and elevated mitochondrial ROS levels can cause mitochondrial dysfunction. We investigated the effects of MitoTempo,[32] a mitochondria-specific superoxide dismutase mimetic, on mitochondrial ROS generation and inflammatory mediator production by dust extract in Beas2B and NHBE cells. We found that MitoTempo suppressed mitochondrial ROS induced by dust extract (Figure 5A–D) but failed to reduce induction of inflammatory mediators except proIL-1β at a relatively high concentration of 20 μM (Figure 5E–I).

3.5 | Effects of inhibition of XO on inflammatory mediators

Metabolism of hypoxanthine to xanthine and uric acid by XO results in the production of O_2^- and H_2O_2.[21] Inhibitors of XO have been shown to have protective effects against respiratory and cardiovascular disorders.[27] We investigated the effects of xanthine oxidase inhibitor, Febuxostat, to determine the involvement of XO pathway in the induction of inflammatory mediators by dust extract. Febuxostat, a potent and specific inhibitor of XO suppressed induction of proIL-1β and ICAM-1 but not IL-6 and IL-8 by dust extract in Beas2B cells (Figure 6A–E) indicating that XO pathway contributes to the induction of inflammatory mediators by dust extract. Febuxostat suppressed dust extract induced DCFDA fluorescence in Beas2B cells indicating inhibition of ROS generation (Figure 6F,G).

3.6 | Effects of VAS2870 on inflammatory mediator levels and 4-HNE immunostaining in in mouse lungs

To gain insights into the contribution of NOX enzymes in dust extract induction of inflammatory mediators in vivo, we determined the effects of VAS2870 on inflammatory mediator expression in mouse lungs. Results of the effects of VAS2870 on inflammatory mediator levels in lung homogenates showed that VAS2870 suppressed dust extract induction of KC, IL-6, and TNF-α but the reduction in IL-1β levels was not significant (Figure 7A–D). These data indicated that as in the case of lung epithelial cells in vitro, NOX enzymes control dust extract induction of inflammatory mediator levels in mouse lungs in vivo. Analysis of 4-HNE, a lipid peroxidation product, by immunostaining showed that 4-HNE staining was increased in the airway epithelium of dust extract treated mice and VAS2870

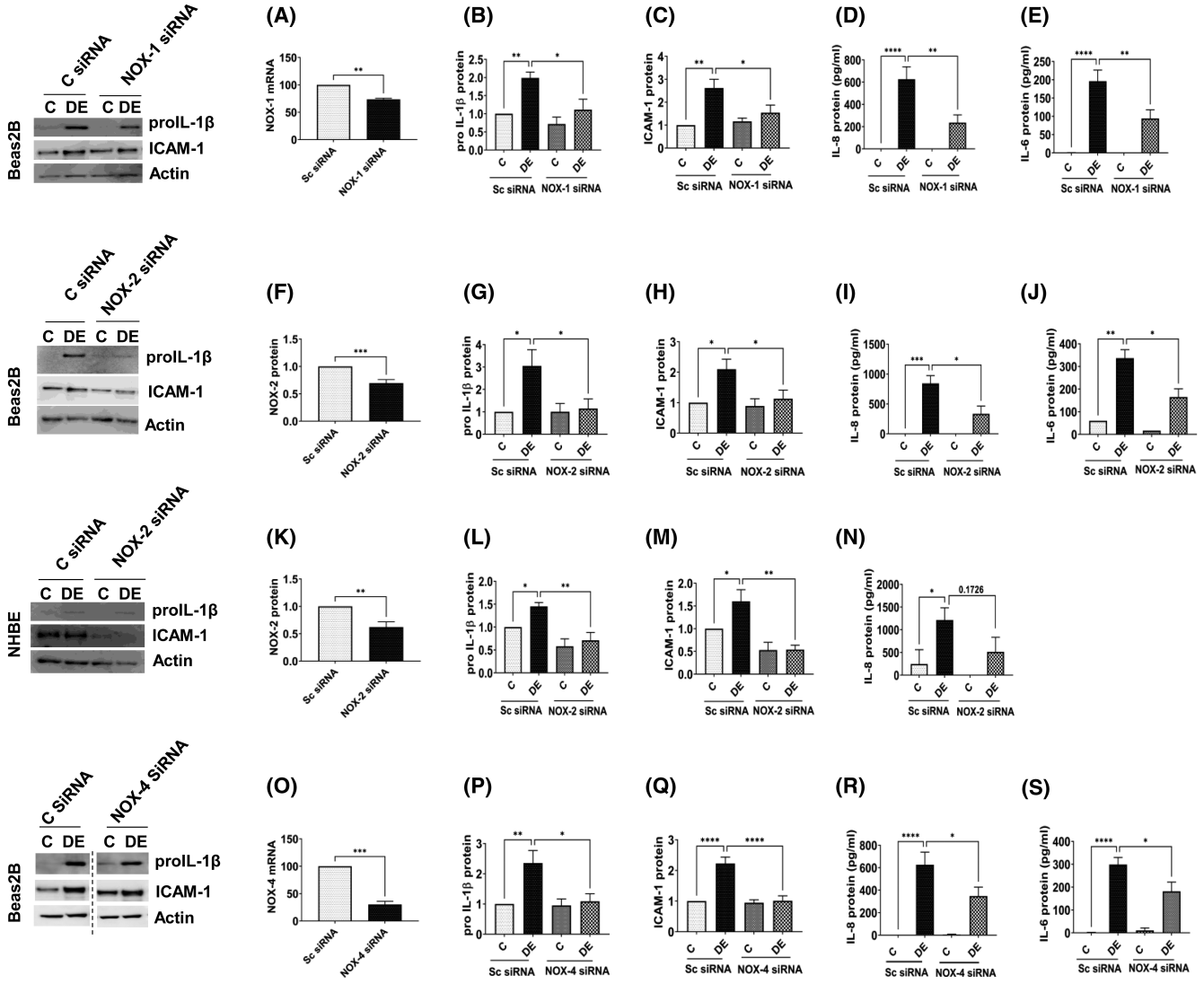

FIGURE 3 Effects of siRNA-targeted knockdown of NOX enzymes on dust extract induction of inflammatory mediators in bronchial epithelial cells. Beas2B (A–J and O–S) and NHBE (K–N) cells were transfected with scrambled (Sc), NOX1, NOX2, or NOX4-targeted siRNA and after 60–72 h of incubation, cells were treated with medium (C) or dust extract (DE) (0.25%) for 3 h. Levels of NOX2, proIL-1β, and ICAM-1 proteins in cell lysates and IL-8 and IL-6 proteins in cell medium were determined. Cellular protein levels were normalized to actin protein levels. Representative western blots are shown. Noncontiguous lanes in western blot image are indicated by a broken line. Levels of NOX1 and NOX4 mRNAs were normalized to 18 S rRNA or actin mRNA levels. Data shown are mean ± SE ($n = 5$–6 for Beas2B cells and $n = 4$ for NHBE cells). *$p < .05$, **$p < .01$, ***$p < .001$.

treatment reduced staining intensity (Figure 7E). These data indicated that NOX-mediated oxidative stress controls dust extract induction of inflammatory mediator expression in mouse lungs.

3.7 | Effects of dust extract on NOX2 protein levels and p47^{phox} activation

Our data indicated that NOX2 plays an important role in dust extract induced ROS generation and inflammatory mediator production. Increased ROS generation could be due to NOX2 activation and/or increased NOX2 expression. To understand mechanisms mediating increased ROS generation by NOX2, we determined the effects of dust extract on NOX2 protein levels and on the activation of p47^{phox}, a protein subunit that controls membrane organization and activation of NOX2.[20] We found that dust extract did not appear to increase NOX2 protein levels (Figure 8A,B) suggesting that perhaps NOX activation leads to elevated ROS generation and inflammatory mediator production. Active NOX is a multi-subunit protein comprising membrane- and cytosolic-subunits organized on the plasma membrane.[20,23] Upon activation, p47^{phox} which normally resides in the cytosol undergoes serine-phosphorylation

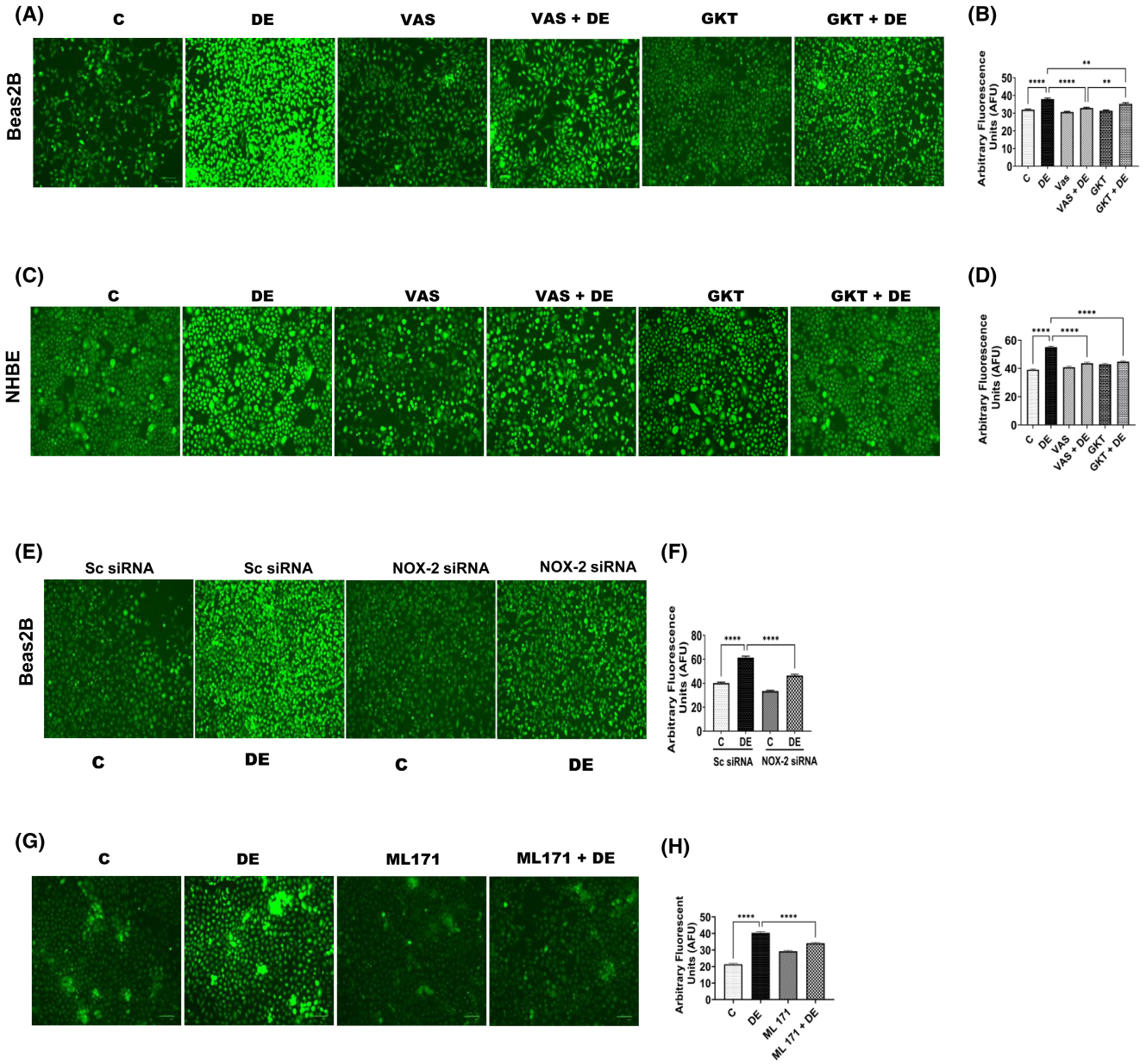

FIGURE 4 Effects of NOX chemical inhibitors and NOX2 knockdown on dust extract induction of ROS generation in bronchial epithelial cells. Beas2B (A,B,E–H) and NHBE (C,D) cells were incubated first with or without VAS2870 (5 μM), GKT137831 (10 μM), or ML171 (10 μM) for 1 h and then treated with medium (C) or dust extract (DE) (1%) for 30 min. (E,F) Beas2B cells were transfected with scrambled (Sc) orNOX-2-targeted siRNA and grown for 60–72 h and then treated with medium (C) or dust extract (DE) (1%) for 30 min. ROS generation was visualized by staining cells with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) and images were captured with a Zoe fluorescent cell imager. Representative images are shown. DCF fluorescence in randomly selected 60 cells from each experiment was quantified using Image J. Data shown are mean ± SE ($n = 3$). ****$p < .0001$.

followed by translocation to the plasma membrane where it organizes the formation of the active NOX enzyme.[20,23] We studied the translocation of $p47^{phox}$ by immunofluorescence confocal microscopy to determine if dust extract treatment activates $p47^{phox}$. We found that treatment of Beas2B cells with dust extract rapidly enhanced translocation of $p47^{phox}$ to the plasma membrane (Figure 8C) implying NOX2 activation that could lead to increased ROS generation.

3.8 | Effects of VAS2870 on NF-κB and Stat3 activation

We have found previously that increased ROS generation by dust extract activates NF-κB[9,19] and Stat3[28] to control induction of inflammatory gene expression in Beas2B cells. We investigated the effects of VAS2870 on NF-κB and Stat3 activation to determine if NOX generated ROS mediate their activation. We found that VAS2870 suppressed

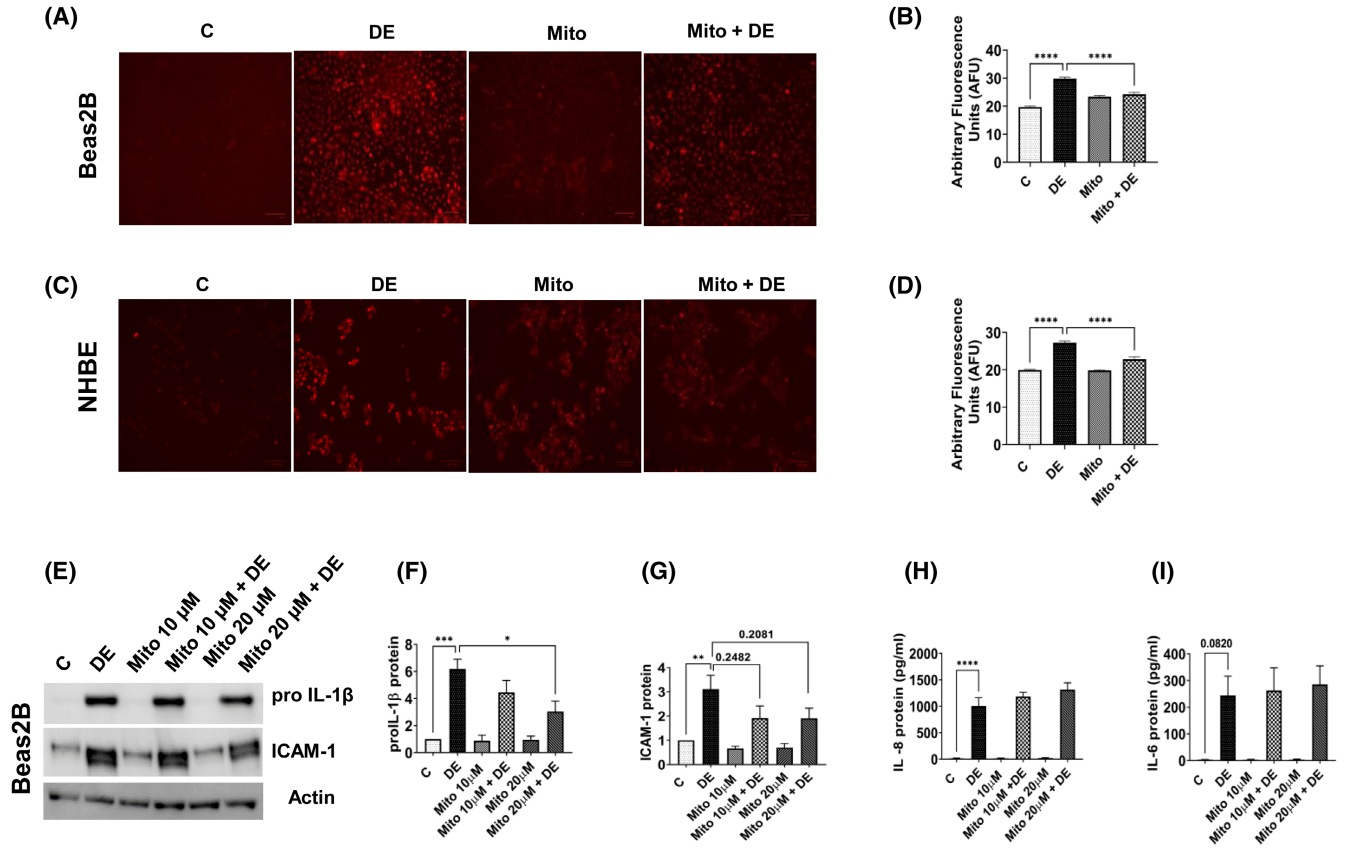

FIGURE 5 Effect of dust extract on mitochondrial ROS generation and effect of MitoTempo on dust extract induction of ROS generation and inflammatory mediators in bronchial epithelial cells. (A–D). Beas2B and NHBE cells were first incubated with or without MitoTempo (Mito) (10 μM) for 1 h and then treated with dust extract (1%) for 1 h. Mitochondrial ROS generation was visualized by staining cells with MitoSOX Red. Representative images are shown. Fluorescence was quantified in randomly selected 60 cells from each experiment using Image J. Data shown are mean ± SE ($n = 4$), $^{****}p < .0001$. (E–I) Beas2B cells were first incubated with the indicated concentrations of MitoTempo (Mito) for 1 h and then treated with medium (C) or dust extract (DE) (0.25%) for 3 h. Levels of proIL-1β, and ICAM-1 proteins in cell lysates and IL-8 and IL-6 proteins in cell medium were determined. Cellular protein levels were normalized to actin protein levels. A representative western blot is shown. Data shown are mean ± SE ($n = 6$). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$.

NF-κB and Stat3 activation induced by dust extract in Beas2B cells (Figure 9A–D). Analysis of Stat3 activation in mouse lungs showed that VAS2870 suppressed increase of phosphoStat3 levels by dust extract (Figure 9E,F). Collectively, these data indicated that increased ROS generation by NOX enzymes mediates NF-κB and Stat3 activation to induce inflammatory mediator expression by dust extract.

4 | DISCUSSION

Mechanisms by which organic dust induces lung inflammatory responses are not fully understood. We found previously that treatment of lung epithelial cells with poultry organic dust extracts increased intracellular ROS levels causing induction of inflammatory mediators that was suppressed by antioxidants such as dimethylthiourea, n-acetylcysteine, and CDDOIm.[19] In this study we show that NOX- and XO-derived ROS contribute to the induction of inflammatory mediators by poultry organic dust and provide evidence for the activation of NOX2 as a mechanism for increased ROS generation. The primary function of membrane-bound NOX enzymes is extracellular production of O_2^- and/or H_2O_2.[20,23] Superoxide anion undergoes dismutation spontaneously and/or by the action of superoxide dismutase to produce hydrogen peroxide in the extracellular space. Extracellular superoxide anion has been reported to traverse cell membrane via chloride channel-3 (CLC3), whereas H_2O_2 crosses cell membrane via aquaporin channel.[33]

Pharmacological inhibitors of NOX have proven useful to understand the contributions of different NOX isoforms, however, their effects must be interpreted with caution as the inhibitors may not be entirely selective.[29,31] Our studies using pharmacological inhibitors and siRNA knockdown approach indicated that NOX1, NOX2 NOX4, and XO are important players in dust extract induction of ROS

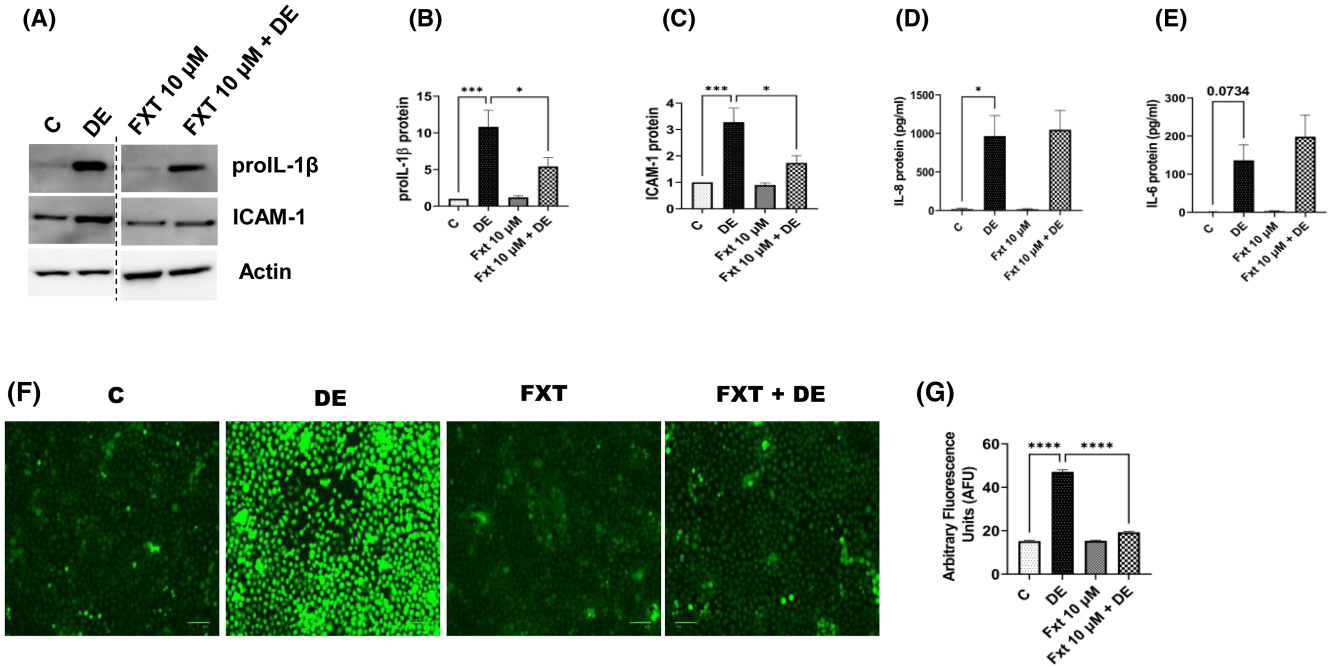

FIGURE 6 Effects of xanthine oxidase inhibitor, febuxostat on dust extract induction of inflammatory mediators and induction of ROS generation in Beas2B bronchial epithelial cells. (A–E) Cells were first treated with febuxostat (10 μM) for 1 h and then incubated with dust extract (DE) (0.25%) for 5 h. Levels of proIL-1β, and ICAM-1 proteins in cell lysates and IL-8 and IL-6 proteins in cell medium were determined. Cellular protein levels were normalized to actin protein levels. Representative western blots are shown. Noncontiguous lanes in western blot images are indicated by a broken line. Data shown are mean ± SE ($n = 5$). *$p < .05$ and ***$p < .001$. (F,G) Cells were first treated with febuxostat (10 μM) for 1 h and then exposed to DE (0.25%) for 30 min. ROS generation was visualized by staining cells with 2′,7′ Dichlorodihydrofluorescein diacetate (DCFDA) and images were captured with a Zoe fluorescent cell imager. Representative images are shown. DCF fluorescence in randomly selected 60 cells from each experiment was quantified using Image J. Data are mean ± SE ($n = 4$). ****$p < .0001$.

generation and inflammatory mediators. Administration of VAS2870 reduced dust extract induction of KC, IL-6 and TNF-α levels and 4-HNE staining in lungs of mice further supporting a role for NOX derived ROS for the induction of inflammatory mediators. NOX 4 is localized in the inner mitochondrial membrane and generates predominately H_2O_2 contributing to the overall ROS levels in mitochondria.[34] We found that NOX4 knockdown inhibited dust extract induction of proIL-1β, ICAM-1, IL-6, and IL-8 (Figure 3O–S) whereas MitoTempo, a mitochondria-specific antioxidant and superoxide dismutase mimetic did not except inhibit proIL-1β at a high concentration (20 μM; Figure 5G). This suggested that although mitochondrial O_2^- is increased by dust extract, it does not appear to play a direct role in the induction of inflammatory mediators.

We have found that increased production of intracellular ROS by dust extract occurs rapidly followed by induction of inflammatory mediators suggesting that activation rather than increased expression of NOX controls induction of inflammatory mediators under acute treatments.[19] Consistent with this interpretation, our studies showed that dust extracts did not increase NOX2 (gp91phox) protein levels but increased membrane association of p47phox indicative of NOX2 activation. Although NOX enzymes share similar structural features, they differ in their mechanism of activation. Intracellular calcium levels or protein–protein interactions control activation of NOX enzymes.[35] All NOX enzymes are subject to regulation at the level of activation with the exception of NOX4 which may be constitutively active even though data suggest that it is activated by agonist stimulation.[35] NOX2 like other NOX enzymes is a multicomponent enzyme complex comprising two membrane proteins, p22 and gp91or NOX2, and four cytosolic proteins, $p47^{phox}$, p67, p40 and Rac2 that assemble at membrane sites after cell activation.[35] NOX2 is inactive in resting cells but is rapidly activated upon cell stimulation. Activation of NOX2 is dependent on the phosphorylation of $p47^{phox}$ at multiple serine residues which occurs by the actions of different types of protein kinases such as protein kinase (PK)Cs, PKA, p38 and p44/42 MAP kinases, interleukin-1 receptor associated kinase (IRAK)-4, src kinase, and others.[35] Upon phosphorylation, conformational changes in p47 promote its interactions with gp91/NOX2, p22 and p67 leading

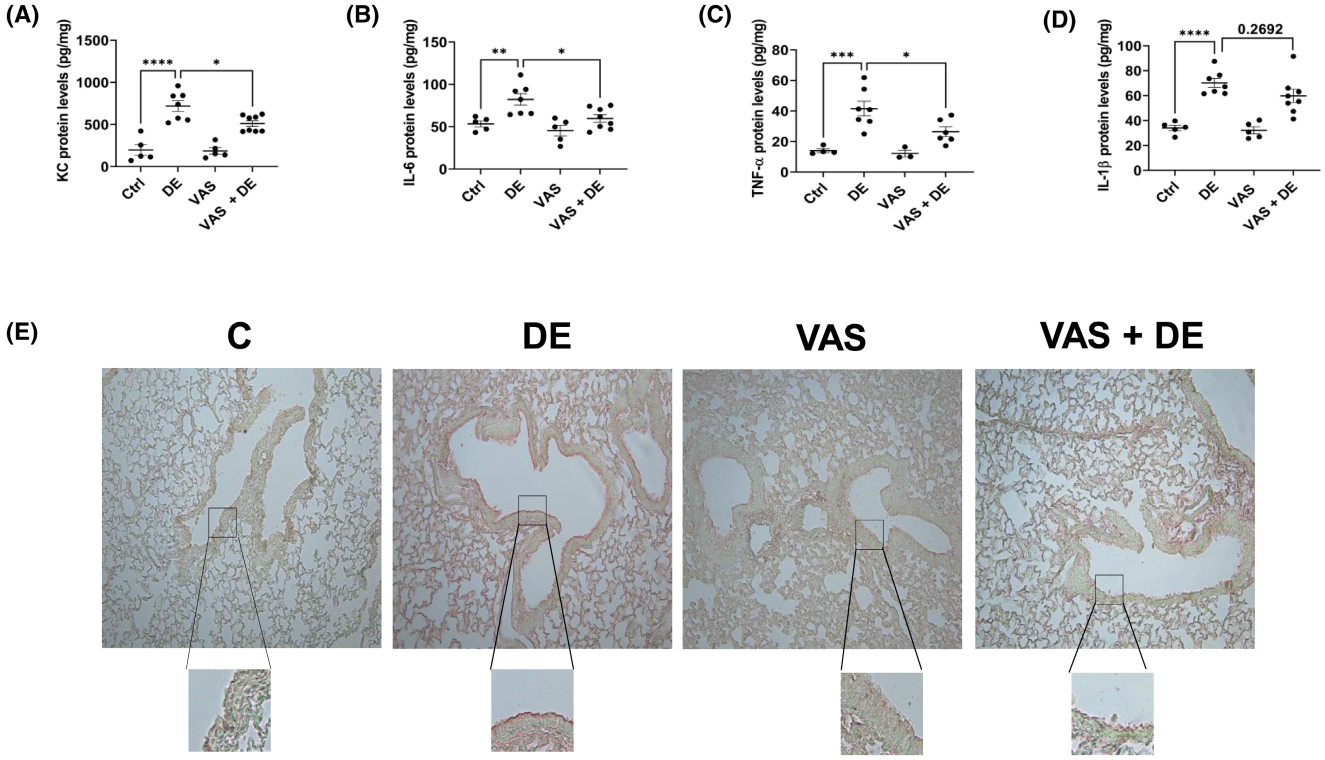

FIGURE 7 Effects of VAS2870 on induction of inflammatory mediators and on 4-hydroxynonenal (4-HNE) staining by dust extract in mouse lungs. Female C57BL/6 mice received vehicle or VAS2870 via intraperitoneal injection 1 h before intranasal administration of 50 μl of PBS (C) or 10% dust extract (DE). After 3 h, mice were euthanized, and lungs were cleared of blood by perfusing with D-PBS and homogenized. (A–D) Levels of keratinocyte chemoattractant (KC), IL-6, TNF-α and IL-1β were determined by ELISA and normalized to total protein levels. Data shown are means ± SE ($n = 5–8$). *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$. (E) Lung sections from mice ($n = 5–6$) were immunostained with 4-HNE antibody and developed using AEC substrate. Representative images are shown at 100 × magnification.

to the assembly of the active NOX enzyme complex at membrane sites.[35]

The components of dust extract responsible for the activation of NOX are not known. Organic dust contains Gram-negative and Gram-positive bacteria and their byproducts such as lipopolysaccharide (LPS) and peptidoglycan.[7,36] We have found that poultry organic dust extracts contain LPS and peptidoglycan,[9] proteases,[19] and bacterial extracellular vesicles derived from Gram-positive and Gram-negative bacteria.[8] We have reported previously that proteases and bacterial extracellular vesicles in dust extracts contribute to induction of inflammatory responses.[8,19] These different constituents of organic dust could potentially activate cell signaling pathways to activate NOX2 to increase ROS production causing induction of inflammatory mediators. Cellular signaling pathways and mechanism of NOX2 activation by dust extract are not known. Cytokines, growth factors, toll-like receptors, lipids, and mechanical stimuli are known to activate NOX enzymes in calcium dependent manner.[35] Toll-like receptor 2 (TLR2) was found to be important for the regulation of airway inflammation in mice treated with extracts of swine facility organic dust suggesting the involvement of TLR2 agonists.[37] In human embryonic kidney HEK293T cells, LPS increases ROS generation and NFκB activation via direct interaction of TLR4 with NOX4.[38] In human patients with neurodegenerative diseases high levels of serum LPS and increased levels of 8-iso-prostaglandin F2α (8-iso-PGF2α), a stable end product of oxidation of arachidonic acid were found to be associated with increased NOX2 activation.[39] Lipoteichoic acid (LTA), a major component of cell wall of Gram-positive bacteria activates or induces heme oxygenase-1 (HO-1) in human tracheal smooth muscle cells,[40] matrix metalloproteinase-9 (MMP-9) in brain astrocytes[41] and MUC5AC in human nasal epithelial cells[42] via activation of NOX enzymes. Upregulation of NOX enzymes has been implicated in the development of human diseases including neurological, cardiovascular, and respiratory diseases.[43] Targeting NOX enzymes selectively rather than targeting global ROS production has been suggested as a better strategy for disease treatment.[44] Our studies have shown that NOX and XO enzymes are important mediators of lung inflammatory responses due to organic dust exposure and could be targeted for the

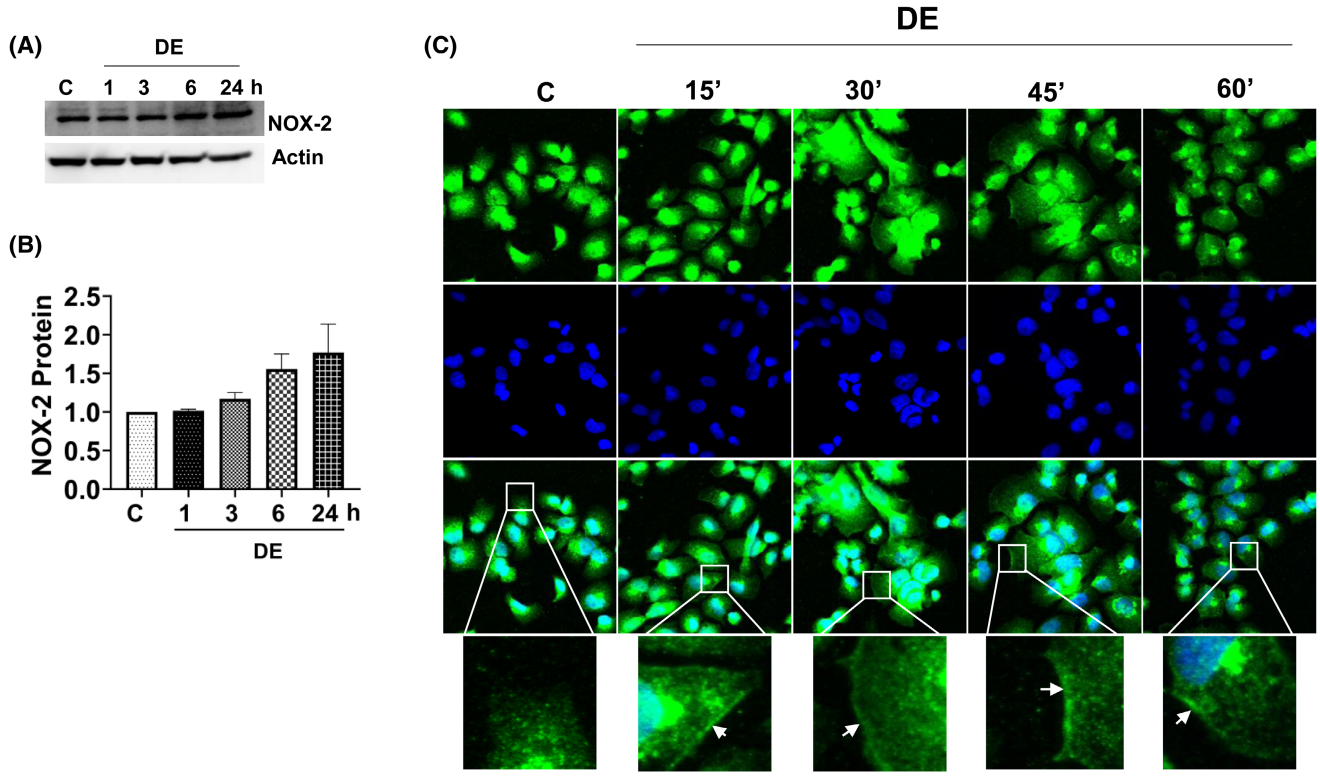

FIGURE 8 Effects of dust extract on NOX2 protein levels and on membrane localization of p47phox. (A,B) Beas2B cells were treated with medium (C) or dust extract (DE) (0.25%) for the indicted times. NOX2 protein levels were determined by western blotting and normalized to actin levels. Representative western blots are shown. Data shown are mean ± SE ($n = 5$). (C) Beas2B cells grown on glass coverslips were treated with medium (C) for 60 min or DE (1%) for 15–60 min. p47phox was visualized by immunostaining with p47phox antibodies followed by Alexa488-labeled secondary antibody. Nuclei were visualized by staining with 4′,6′-diamidino-2-phenylindole (DAPI). Representative confocal fluorescence microscopic images are shown. Green—p47phox staining, blue—nuclei staining. Increased membrane localization of p47phox is indicated by the arrows.

treatment of occupational respiratory diseases among agricultural workers.

The production of ATP by the mitochondrial electron transport chain generates ROS as byproducts.[45] Basal levels of ROS serve to integrate mitochondrial function with cellular function, but elevated mitochondrial ROS can impair mitochondria leading to mitochondrial dysfunction.[45] Indeed, elevated mitochondrial ROS are implicated in the development of mitochondrial diseases.[46] Electron transport chain complex I and III are believed to be the major sites for the production of ROS. Inhibition of complex I due to inactivation and/or altered expression of proteins that make up complex I results in elevated levels of ROS causing mitochondrial oxidative stress and mitochondrial dysfunction. Crosstalk between ROS derived from NOX and that produced by mitochondria termed "ROS induced ROS release" that results in a feed-forward amplification of ROS generation has been implicated in different pathologies.[47] Our data indicated that elevated mitochondrial O_2^- may not directly contribute to dust extract induction of inflammatory mediators whereas NOX4-derived H_2O_2 is important. ROS derived from XO appeared to be important for the induction of proIL-1β and ICAM-1. The effects of XO inhibitor febuxostat to suppress dust extract induction of proIL-1β and ICAM-1 levels could be partly mediated via inhibition of NOX enzymes as it is reported to inhibit NOX activity.[48]

We have found that dust extract activates NF-κB, AP-1, and Stat3 transcription factors in a redox-sensitive manner to induce inflammatory mediator expression.[19] In this study, we found that VAS2870 inhibited NF-κB and Stat3 activation by dust extract indicating that NOX-derived ROS control their activation. NF-κB[49] and Stat3[50,51] are redox-sensitive transcription factors that are subject to oxidative stress mediated activation or inhibition depending on the cellular context. Both classical and atypical pathways are activated by oxidative stress to activate IκB kinases (IKK) to increase phosphorylation of IκBα thereby promoting its degradation and activation of NF-κB.[49] It is also known that oxidative stress inhibits NF-κB activation by direct oxidation of a cysteine residue (cys-62) in the RHD domain of p50 subunit.[49] Inactivation of IKK by direct oxidation by ROS

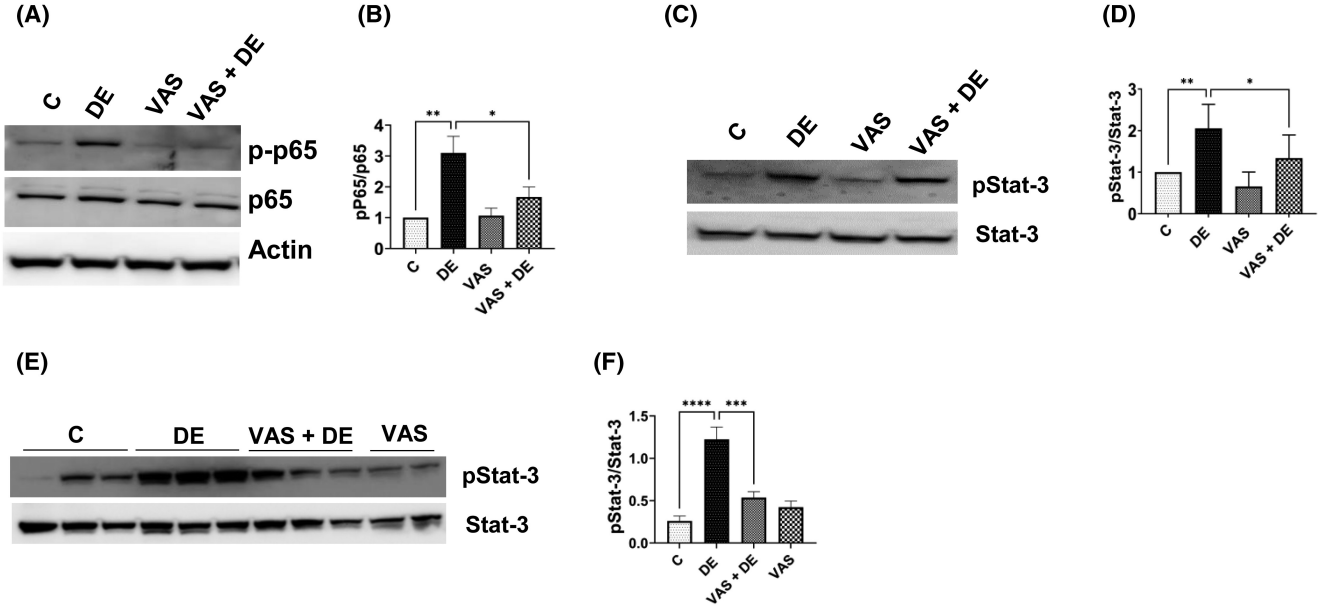

FIGURE 9 Effects of VAS2870 on NF-κB and Stat3 activation by dust extract. (A–D) Beas2B cells were first incubated with medium (C) or VAS2870 (5 μM) for 1 h and then treated with dust extract (DE) (1%) for 10 min to determine NFκB activation and for 1 h to determine Stat3 activation. Levels of phospho-p65 and phospho-Stat3 were normalized to total p65 and total Stat3 levels respectively. Data shown are mean ± SE ($n = 5$–6). *$p < .05$, **$p < .01$. (E,F) Mice received vehicle or VAS2870 1 h before intranasal instillation of 50 μl of D-PBS (C) or 10% DE. After 3 h, phospho-Stat3 levels in lung homogenates were determined and normalized to total Stat3 levels. A western blot showing pStat3 and Stat3 levels in lungs of 3 mice from C, DE, and VAS + DE and 2 mice from VAS groups is shown. Data shown are mean ± SE ($n = 5$ for control and VAS treated mice, $n = 8$ for DE and VAS + DE treated mice). ***$p < .001$, ****$p < .0001$.

leading to inhibition of NF-κB activation has also been reported.[49] Oxidative stress modulates tyrosine kinases such as JAK and Src kinases and tyrosine phosphatases to control Stat3 activation by phosphorylation.[52] On the other hand, oxidative stress can directly modify JAK2[53] and Stat3[54] by oxidation of critical cysteine residues to inhibit Stat3 activation. Our previous studies indicated that Tyk2 and EGFR tyrosine kinases mediate Stat3 activation by dust extract.[28] Further studies are necessary to understand the mechanisms by which NOX-derived ROS controls dust extract activation of NF-κB and Stat3 in airway epithelial cells.

Our studies used bronchial epithelial cells grown under submerged culture conditions which is a limitation as they do not polarize to mimic the phenotype of the bronchial epithelial cell. However, our findings from cell culture studies were in agreement with findings from mouse studies supporting the relevance of cell culture findings. Our studies did not address the contributions of ROS generated from NOX3, NOX5, DUOX1, and DUOX2 in the induction of inflammatory mediators and were limited to airway epithelial cells. Future studies should address the roles of these other NOX enzymes and the roles of NOX enzymes in alveolar epithelial cells and immune cells such as macrophages and neutrophils to fully understand lung inflammatory outcomes elicited by organic dust.

In summary, our studies showed that elevated levels of ROS generated by NOX and XO but not mitochondria control induction of lung inflammatory mediators by organic dust extract. Activations of NFκB and Stat3 by NOX generated ROS were found to mediate induction of inflammatory mediator expression. Targeting NOX and XO enzymes could serve as a therapeutic strategy to reduce lung inflammation in respiratory diseases afflicting agricultural workers.

AUTHOR CONTRIBUTIONS

Vijay Boggaram conceived and wrote the manuscript. Velmurugan Meganathan, Cory E. Hamilton, Kartiga Natarajan, and Vijay Boggaram designed and performed the experiments, analyzed, and interpreted the data. Shiva Keshava captured confocal microscopy images and interpreted them. Vijay Boggaram supervised the study, and all authors approved the final manuscript.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study can be found in the paper and in its supplementary materials published online.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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