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RESEARCH ARTICLE

Purification and characterization of a serine protease from organic dust and elucidation of its inductive effects on lung inflammatory mediators

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Abstract

Organic dust inhalation is associated with the development of respiratory diseases. Serine protease activities in organic dusts were previously reported to contribute to the induction of lung inflammatory mediators however, the identities of the proteases and the mechanisms by which they induce inflammatory mediators are unknown. The goal of this study was to purify and characterize serine protease(s) from organic dust and elucidate mechanisms by which they induce lung inflammatory mediators. A serine protease was purified from poultry organic dust by benzamidine-agarose affinity chromatography. Mass spectrometry and amino-terminal sequence analysis identified the purified protease as chicken trypsin II-P29. Purified protease induced proinflammatory cytokine levels in Beas2B and NHBE epithelial and THP-1 macrophage cells. Treatment with the purified protease increased cellular and mitochondrial reactive oxygen species (ROS) generation. Induction of inflammatory mediators and ROS were suppressed by serine protease inhibitors and antioxidants. Purified protease activated protein kinase C (PKC), mitogen-activated protein kinase (MAPK)1/3 and MAPK14 signaling, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (Stat-3), and chemical inhibitors targeting these pathways suppressed induction of inflammatory mediators. Calcium mobilization studies showed that the purified protease activated protease-activated receptors (PAR) F2R, F2RL1, F2RL2, F2RL3, and F2R and F2RL1 knockdown suppressed the induction of inflammatory mediators. Intranasal instillation of purified protease increased lung chemokine (C-X-C motif) ligand (CXCL)1, interleukin (IL)-6, and tumor necrosis factor (TNF) levels in mice. Our studies have shown that chicken trypsin is a proinflammatory constituent of poultry organic dust, and induces lung inflammatory mediators via increased ROS and PAR activation in a cell signaling pathway involving PKC, MAPK1/3 and MAPK14, and NF-κB and Stat-3.

NEW & NOTEWORTHY Inhalation of dust in industrial agricultural operations is linked to the development of lung diseases. Our studies have isolated for the first time a trypsin protease from poultry farm dust and have shown that it stimulates lung inflammation. The protease stimulates the production of oxidants and cell signaling pathways to increase inflammatory mediator production. Targeting trypsin protease in poultry farm environment may be a useful strategy to counter the harmful effects of dust.

agricultural dust; cytokines; gene regulation; reactive oxygen species; trypsin

INTRODUCTION

Industrial animal farming known as concentrated animal feeding operations (CAFO) exposes workers to high levels of airborne dust thereby increasing their risk for the development of respiratory diseases, such as chronic bronchitis, asthma, hypersensitive pneumonitis, and chronic obstructive pulmonary disease (COPD) (1). In particular, the prevalence of respiratory symptoms and lowered lung function parameters are higher among poultry workers than in other agricultural workers (2, 3). Although it is well known that organic dust exposure is linked to the development of respiratory diseases, there is a lack of information on dust constituents and mechanisms by which they promote disease pathogenesis.

Organic dust is a complex matter derived from materials found in animal farms such as bedding, feed, dander, feathers, microbes, and microbial products as well as methane, ammonia, and hydrogen sulfide gases that are produced during the decomposition of animal waste (1, 4). Treatment of airway epithelial cells, macrophages, and monocytic cell lines with different organic dust extracts induces the expression and secretion of cytokines such as interleukin (IL)-6, chemokine (C-X-C motif) ligand (CXCL) 8 (IL-8), and tumor necrosis factor (TNF) (5–8). Similarly, mice exposed to swine and poultry organic dust extracts showed increases in lung CXCL1, IL-6, and TNF levels and inflammatory cell counts (5, 9, 10). Induction of inflammatory gene expression by poultry organic dust extract is mediated via activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signaling pathways and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (Stat-3) transcription factors

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(10, 11). In naïve human subjects, as little as 2–5 h of exposure to the swine barn environment induced bronchial responsiveness, fever, and malaise (12, 13). These symptoms were associated with increases in neutrophil, eosinophil, and lymphocyte counts in nasal lavage and bronchoalveolar lavage (BAL) fluid (14). Neutrophil counts increased by greater than 50-fold, whereas alveolar macrophage and lymphocyte counts increased by two- to threefold (15).

Although organic dust contains several potential proinflammatory constituents, those responsible for the induction of lung inflammatory responses are only beginning to be identified. Lipopolysaccharide (LPS) is found abundantly in organic dusts however, it is not clear if it acts independently to induce inflammatory responses (8, 11). We recently reported that bacterial extracellular vesicles isolated from poultry organic dust induce lung inflammatory responses (16). Proteases in organic dusts were recently reported to induce lung inflammatory responses (17, 18), however, there is no information on their identities and the underlying mechanisms. To fill gaps in our knowledge on organic dust-derived proteases, we purified and characterized a serine protease from poultry organic dust by affinity chromatography and studied mechanisms mediating its effects on the induction of lung inflammatory mediators. Some of the data reported here have been presented in the form of a conference abstract (19).

MATERIALS AND METHODS

Chemicals and Reagents

Tempol (Tocris) and dimethylthiourea (DMTU) (Acros Organics) were used as antioxidants. 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) (EMD Millipore), *p*-aminobenzamidine (Sigma), leupeptin (Enzo Life Sciences), α1-antitrypsin (Athens Research and Technology), soybean trypsin inhibitor (SBTI) (Sigma), and protease inhibitor cocktail (PIC) (Thermo Fisher Scientific) were used as protease inhibitors. NF-κB inhibitor BAY 11-7082 (Santa Cruz Biotechnology), Stat-3 inhibtior Stattic (Tocris), PKC inhibitor bisindolylmaleimide I (Cayman Chemical), MAPK1/3 inhibitor U0126 (Calbiochem), MAPK8/9 inhibitor SP600125 (EMD Millipore), and MAPK14 inhibitor SB203580 (LC Laboratories) were used to inhibit signaling pathways. Proteinase-activated receptor 1 (F2R) agonist peptide (TFLLRN) (Bio-Synthesis, Inc.), proteinase-activated receptor 2 (F2RL1) agonist peptide (SLIGRL) (Bio-Synthesis, Inc.), thrombin (Enzyme Research) and human trypsin (ProSpec) were used as activators of protease-activated receptors (PARs).

Dust Extract Preparation

Dust settled on vertical surfaces inside poultry farms located in East Texas region was obtained with permission and stored at −70°C. Aqueous extract of dust was prepared using endotoxin-free Dulbecco's phosphate-buffered saline (D-PBS) (Lonza) without calcium and magnesium as described previously (11). Briefly, dust was suspended in D-PBS (1:10, wt/vol) and mixed overnight at 4°C using a rotating mixer. Dust suspension was centrifuged twice at 800 *g* for 10 min at 4°C, and the resulting supernatant was centrifuged again at 10,000 *g* for 10 min at 4°C. The supernatant was filtered with a 0.22-μm syringe filter and filtrate stored at −70°C. In some instances, supernatant obtained after isolation of bacterial extracellular vesicles (16) was used for the purification of the protease.

Affinity Chromatography

Dust extract (10 mL) was mixed with 1 mL of Benzamidine Separopore 6B gel suspension (bioWORLD) for 6 h using a rotating mixer at room temperature. Afterward, the suspension was centrifuged for 1 min at 1,500 *g* to pellet the gel, and the gel was washed twice with 5 mL of D-PBS for 15 min at room temperature using a rotating mixer. After washing, the bound protease was eluted by incubating the gel with 1 mL of 20 mM *p*-aminobenzamidine in D-PBS for 45 min on a rotating mixer at room temperature. After elution, *p*-aminobenzamidine was removed and the protease was concentrated with a centrifugal filter (10 kDa MWCO) (Amicon). Protein concentration of purified protease was determined by Bradford assay with bovine serum albumin (BSA) as the protein standard. This procedure was scaled-up proportionately to purify protease from larger volumes of dust extract.

Assay of Trypsin Activity

Trypsin activity of dust extract and purified protease was determined with chromogenic *p*-nitroanilide substrate, Na-benzoyl-D, L-arginine 4-nitroanilide hydrochloride (BAPNA) (Sigma) as described previously (17).

Gel Electrophoresis

Purified protease was analyzed by SDS-polyacrylamide gel electrophoresis on 10% Bis-Tris gels (Life Technologies) and protein bands were visualized by rapid silver staining according to the published protocol (20). Purified protease was also analyzed by zymography on 10% Tris-glycine polyacrylamide gels containing 10% gelatin (ThermoFisher Scientific) and the gel was stained using Colloidal Blue Staining kit (Invitrogen, ThermoFisher Scientific).

Mass Spectrometry

Purified protease was subjected to SDS-PAGE under reducing conditions and stained with Coomassie R-250 stain. The gel was destained and the protein band was excised and sent to Proteomics Core Laboratory, UT Southwestern Medical Center, Dallas, TX, for protein identification by mass spectrometry.

Cell Culture

Beas2B (ATCC CRL 9609) and normal human bronchial epithelial (NHBE) cells (Lonza or Lifeline Cell Technology) were grown on plastic culture dishes under submerged culture conditions. Beas2B cells were also grown at air-liquid interface (ALI) on transwell inserts. For culturing Beas2B cells, plastic dishes and transwell inserts were coated with fibronectin, bovine type 1 collagen, and BSA. Beas2B cells were grown in LHC-9 medium (Invitrogen), bronchial epithelial growth medium (BEGM) (Lonza), or BronchiaLife Epithelial Airway Medium (Lifeline Cell Technology), whereas NHBE cells were grown in BEGM or BronchiaLife Epithelial Airway Medium. Beas2B and NHBE cells when 80–90% confluent were incubated overnight in RPMI 1640 serum-free medium containing antibiotics and antimycotics and subjected to treatments.

For ALI cultures, ~100,000 Beas2B cells were seeded on the apical chamber of transwell inserts (24-well) in 100 μL BEGM/LHC-9 medium with 500 μL of the medium added to the basolateral chamber. After 2 days, medium from the apical chamber was removed exposing the cells to air (airlift) whereas the medium in the basolateral chamber was replaced with fresh medium. After 48 h of airlift, medium in basolateral chamber was removed, rinsed once, and replaced with 500 μL of RPMI medium without serum. Cells were treated by adding 25 μL of RPMI serum-free medium containing purified protease to the apical chamber followed by gentle rocking for 10 min in the cell culture hood and returned to the incubator. After incubation, medium in basolateral chamber was collected and cells were rinsed twice with 100 μL of serum-free RPMI 1640 medium and washes combined for cytokine analysis. Cells were lysed in 100 μL of 1× LDS sample buffer (Invitrogen) containing DTT and the lysate was repeatedly passed through a 27-gauge needle to shear DNA and reduce the viscosity of the lysate.

THP-1 monocytes (ATCC TIB-202) were grown in RPMI 1640 medium containing 0.05 mM β-mercaptoethanol (Sigma-Aldrich), 10% fetal bovine serum (FBS), and antibiotics and antimycotics (THP-1 medium). THP-1 monocytes were differentiated into THP-1 macrophages with 200 nM phorbol 12-myristate 13-acetate (LC laboratories) as described previously (21). Differentiated THP-1 macrophages were incubated in RPMI 1640 serum-free medium containing antibiotics and antimycotics for 3 h before treatments.

Cell Viability Assay

Cell viability was assessed by MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega) according to the manufacturer's protocol.

Determination of Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) levels were detected with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Sigma-Aldrich). Beas2B and NHBE cells when 60–70% confluent were serum starved overnight in RPMI medium without phenol red. Cells were treated with purified protease for 30 min followed by incubation with 10 μM DCFDA for 30 min. Cells were washed twice with PBS warmed to 37°C, and images were captured with ZOE Fluorescent Cell Imager (Bio-Rad). Fluorescence intensities of 60 randomly selected cells from each experiment were quantified using ImageJ (NIH) software.

Determination of Mitochondrial ROS

Mitochondrial ROS generation was detected with MitoSOX Red (Invitrogen). Cells grown to 60–70% confluence were serum starved overnight in RPMI medium without phenol red and treated with the purified protease for 1 h followed by incubation with 5 μM MitoSOX Red for 30 min. After incubation with MitoSOX Red, cells were washed twice with PBS warmed to 37°C and fluorescence intensities were captured with ZOE Fluorescent Cell Imager (Bio-Rad). Fluorescence intensities of randomly selected 60 cells from each experiment were quantified using ImageJ (NIH) software.

RNA Isolation and Quantitative Real-Time RT-PCR

Total RNA from cells was isolated using TRI-Reagent (Molecular Research Center) and RNA purity was checked by analyzing A260/A280 and A260/A230 ratios. For the determination of mRNA levels, total RNA was first treated with DNase (Turbo DNA-free kit, Ambion) to remove genomic DNA contamination and cDNA was synthesized using iScript reverse transcription kit (Bio-Rad). Levels of mRNAs were quantified by real-time qRT-PCR by TaqMan Assay using CFX96 real time PCR detection system (Bio-Rad) and normalized to actin mRNA. The normalized gene expression data (ΔΔ Ct) relative to the control sample arbitrarily assigned as 1 was obtained using CFX Manager Software. TaqMan gene expression assay IDs for measurement of mRNA levels are listed in Table 1.

Enzyme-Linked Immunosorbent Assay

The levels of IL-6 and IL-8 in cell culture medium and IL-6, CXCL1, TNF, and IL-1β levels in BAL fluid were measured by ELISA (R&D Systems) according to the manufacturer's instructions.

Western Immunoblotting

Equal amounts of proteins (20–30 μg) were separated on 10% Bis-Tris gels (NuPAGE) (Life Technologies) alongside protein molecular weight markers using MOPS SDS buffer (NuPAGE) (Life Technologies). Separated proteins were transferred to Hybond-PVDF membranes (0.2 μm) (GE Healthcare) by electroblotting using transfer buffer (NuPAGE) (Life Technologies). Protein bands were visualized by the enhanced chemifluorescence (ECF) or enhanced chemiluminescence (ECL) method using ChemiDoc MP imaging system (Bio-Rad). Membranes were re-probed for actin to assess for equal loading and transfer of proteins. Protein bands were quantified (ImageLab, Bio-Rad) and normalized to actin levels. Levels in control samples were arbitrarily assigned as 1 and levels in treated samples are shown relative to the control sample. Antibodies used and their dilutions are listed in Table 2.

Intracellular Ca^{2+} Mobilization Measurements

PAR activation was analyzed by measuring changes in intracellular Ca^{2+} levels by live cell confocal imaging according to the method described previously (22). Beas2B cells grown on chambered coverglass slides (Lab-Tek) to ~60% confluence were washed twice with buffer A (10 mM HEPES, 150 mM sodium chloride, 4 mM potassium chloride, 11 mM glucose adjusted to pH 7.4) and incubated with 4 μM Fluo 4-AM (Tocris) in buffer B (5 mM calcium chloride dihydrate, 1

Table 1. *List of TaqMan assays*

Target Gene	Assay ID
IL-8	qHsaCEP0053894 (Bio-Rad)
IL-6	qHsaCEP0051939 (Bio-Rad)
IL-1β	qHsaCIP0033362 (Bio-Rad)
ICAM-1	qHsaCEP0024986 (Bio-Rad)
TSLP	Hs00263639_m1 (Thermo Fisher)
F2R	Hs00169258_m1 F2R (Thermo Fisher)
F2RL1	Hs00608346_m1 F2RL1(Thermo Fisher)
Actin	qHsaCEP0036280 (Bio-Rad)

Table 2. *List of primary and secondary antibodies*

	Antibody Dilution	Catalog Number and Company
Primary antibody		
IL-1β (D3U3E) rabbit mAb	1:1,000	12703, Cell Signaling Technology
ICAM-1 (G-5)	1:1,000	sc-8439, Santa Cruz Biotechnology
Anti-β-actin antibody (C-4)	1:1,000	sc-47778, Santa Cruz Biotechnology
Phospho-p38 MAPK (Thr180/Tyr182)	1:1,000	9211, Cell Signaling Technology
p38 MAPK (D13E1) XP rabbit mAb	1:1,000	8690, Cell Signaling Technology
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) rabbit mAb	1:1,000	4377, Cell Signaling Technology
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	1:1,000	4695, Cell Signaling Technology
Phospho-PKC (pan) (βII Ser660) antibody	1:1,000	9371, Cell Signaling Technology
Phospho-NF-κB p65 (Ser536) (93H1) rabbit mAb	1:1,000	3033, Cell Signaling Technology
NF-κB p65 (C-20)	1:1,000	sc-372, Santa Cruz Biotechnology
Phospho-Stat3 (Tyr705) (D3A7) XP rabbit mAb	1:1,000	9145, Cell Signaling Technology
Stat3 (79D7) rabbit mAb	1:1,000	4904, Cell Signaling Technology
Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) rabbit mAb	1:1,000	4668, Cell Signaling Technology
SAPK/JNK antibody	1:1,000	9252, Cell Signaling Technology
Secondary antibody		
Anti-mouse IgG, HRP-linked antibody	1:5,000	7076, Cell Signaling Technology
Anti-rabbit IgG, HRP-linked antibody	1:5,000	7074, Cell Signaling Technology
Anti-rabbit IgG, AP-linked antibody	1:5,000	7054, Cell Signaling Technology
Anti-mouse IgG, AP-linked antibody	1:5,000	7056, Cell Signaling Technology

mM magnesium chloride hexahydrate, 1 mg/mL bovine serum albumin in buffer A adjusted to pH 7.4) for 1 h. Cells were washed twice with buffer B before treatments and changes in fluorescence intensities (excitation/emission: 488 nm/515 nm) were recorded with a confocal microscope (Zeiss) equipped with a ×20 objective. A typical experiment consisted of recording cells for 135 cycles; each cycle for a duration of 3 s. In desensitization experiments, Ca^{2+} signaling was recorded every 3 s for 500 cycles. During the entire course of imaging, cells were maintained at 37°C with a constant supply of 5% CO_2.

Animals

Animal experimentation had previously been approved by the Institutional Animal Care and Use Committee (IACUC). Female C57BL6 mice (10–14 wk, 19–20 g weight) (Jackson Laboratory) were maintained under standard housing conditions of 12:12-h light-dark cycles and fed standard mouse diet and water. Mice were acclimatized for at least 1 wk before treatments. Mice were anesthetized with ketamine and xylazine (100: 8.5 mg/kg ip), and 50 μL of PBS or 50 μL of PBS containing purified protease was administered via intrainasal instillation into mice. Mice were euthanized and BAL fluid was obtained as described previously (16).

siRNA Knockdown

Scrambled [Mission siRNA universal negative control No. 1 (SIC001)], F2R (SASI_Hs01 00240436/F2R) and F2RL1 (SASI_Hs02 00339249/F2RL1) siRNAs were obtained from Millipore Sigma. siRNAs were transfected into Beas2B cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions, and cells were grown for 48–72 h. Cells were washed twice with RPMI medium without serum and subjected to treatments.

Statistical Analysis

All experiments were performed at least three times independently and the data are shown as means ± SE. Statistical significance between the two groups was analyzed by a two-tailed unpaired *t* test and that between multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 9.

RESULTS

Purification and Characterization of Serine Protease(s) from Poultry Organic Dust

A serine protease from poultry organic dust was purified and characterized by affinity chromatography using benzamidine separopore 6B (23). Purification by affinity chromatography resulted in homogenous pure protein with a yield of ~2.3%. Analysis of the purified protease by SDS-PAGE under reducing conditions and silver staining produced a single protein band of ~26 kDa and a minor band slightly lower in size (Fig. 1*A*). The minor band was not detected in all purified protease samples (data not shown). Protease purified from dust samples collected from two different poultry farms in East Texas displayed similar or identical size (Fig. 1*A*). Analysis by SDS-PAGE under reducing and nonreducing conditions produced a single band of ~25 kDa indicating that the purified protease is composed of a single polypeptide chain (Fig. 1*B*). The purified protease displayed trypsin-like activity. Trypsin-like activity was inhibited by heat treatment and by serine-protease inhibitors such as benzamidine, leupeptin, SBTI, PIC, and AEBSF but not by α1-antitrypsin (Fig. 1*C*). Analysis by gelatin zymography resulted in a single protein band of ~17 kDa and the activity of the band was inhibited by heat treatment, benzamidine, leupeptin, SBTI, and PIC but not by α1-antitrypsin (Fig. 1*D*).

Mass spectrometry analysis pointed to the identity of the purified protein as *Gallus gallus* (chicken) trypsin II-P29 (Accession Number: Q90629) having a molecular weight of 26.6 kDa based on its highest abundance ($6.69e+09$), coverage (58%), peptide spectral matches (PSMs) (323), and unique peptides numbering to 15. The

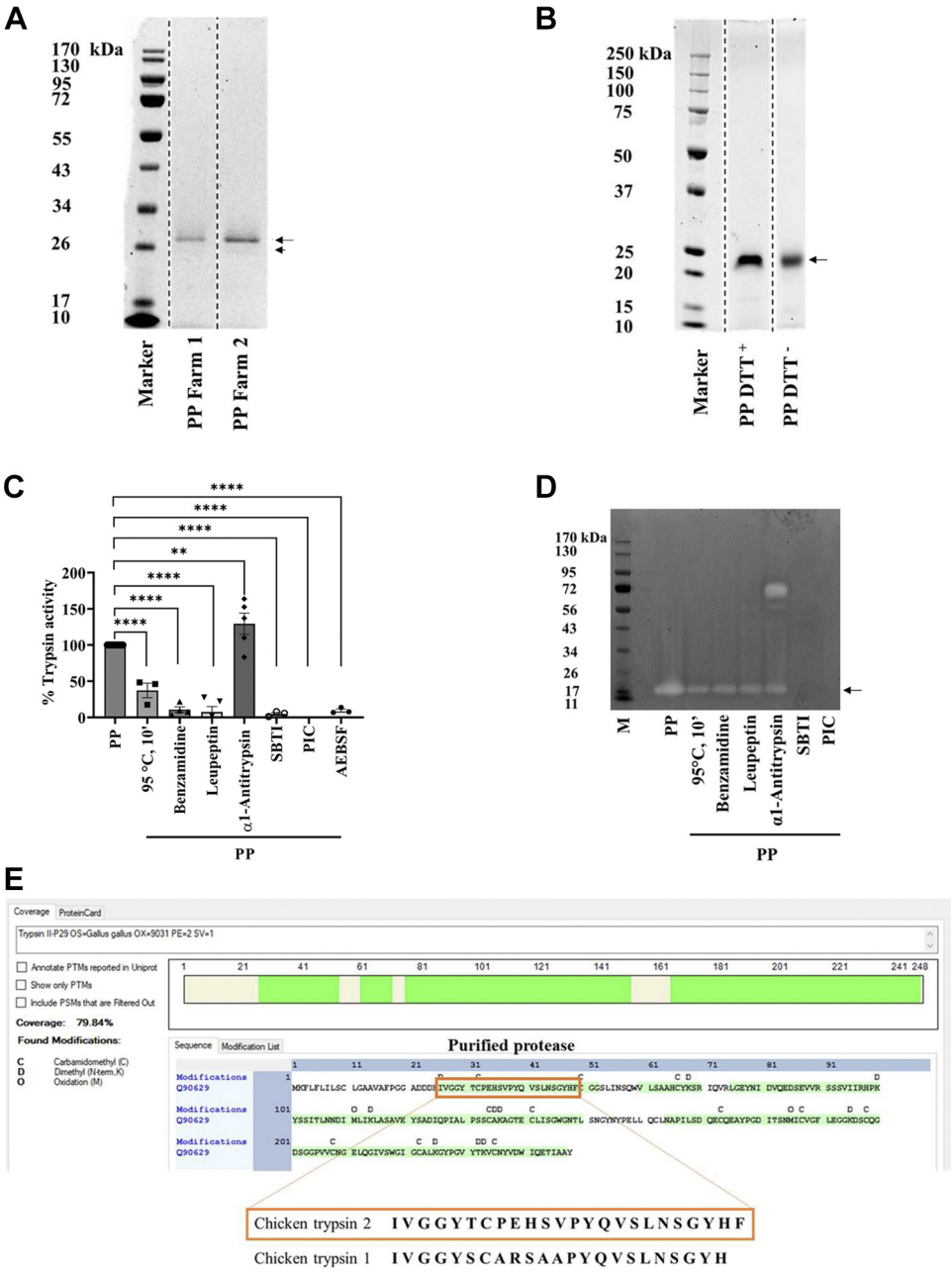
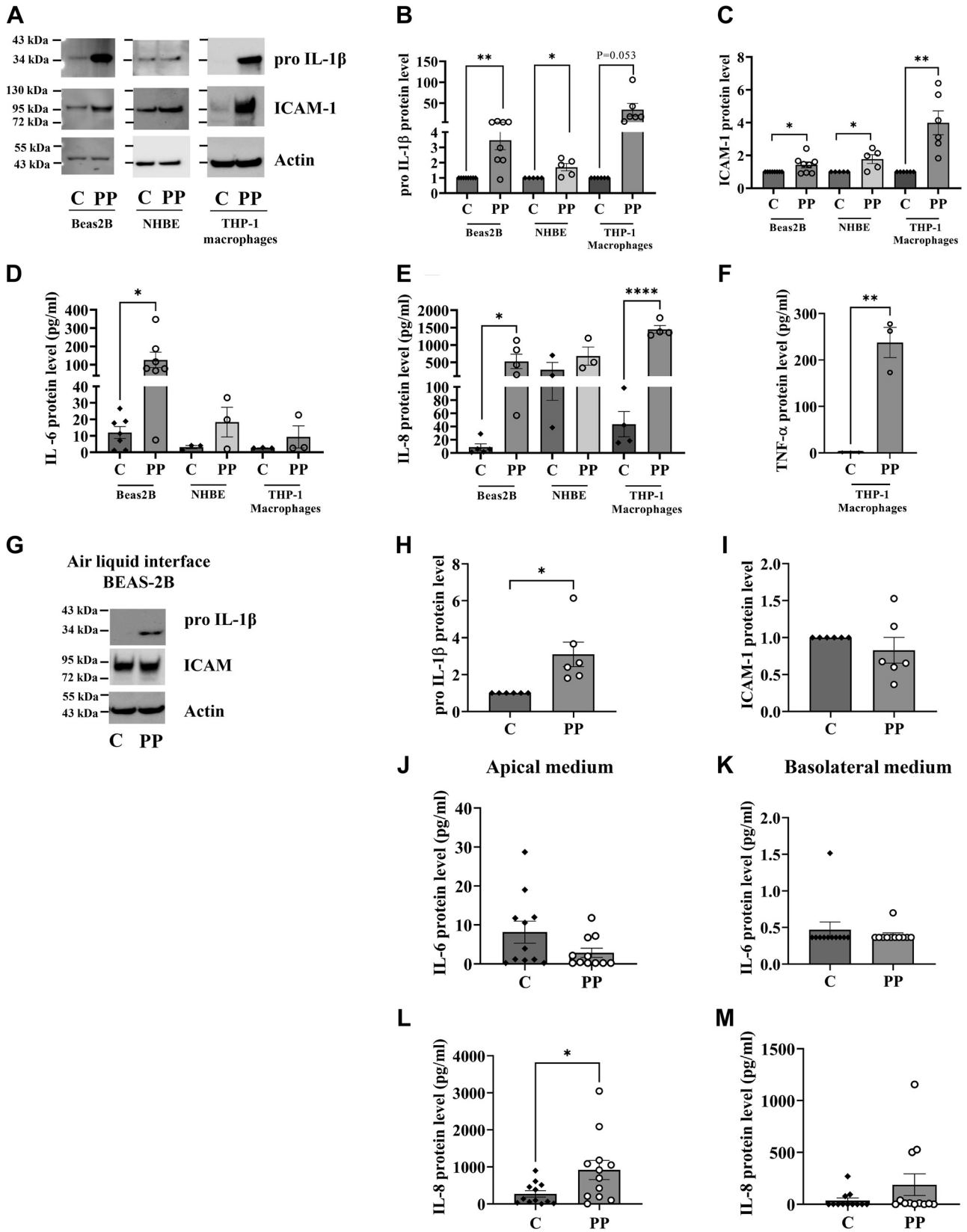


Figure 1. Characterization of the purified protease. *A*: protease purified from dust collected from two different poultry farms was analyzed by SDS-PAGE and silver nitrate staining. A single major protein band of ~26 kDa was observed in both samples (arrow) while a minor band was also evident (arrow head). *B*: purified protease was analyzed by SDS-PAGE with and without dithiothreitol (DTT) and gel stained with coomassie brilliant blue. Noncontiguous lanes in gel are demarcated by broken lines. *C* and *D*: the effects of heat inactivation (10 min at 95°C) and serine protease inhibitors [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 mM *p*-aminobenzamidine, 20 μM leupeptin, 25 μg/mL α1-antitrypsin, 25 μg/mL soya bean trypsin inhibitor (SBTI), and 2× protease inhibitor cocktail (PIC)] on trypsin activity of the purified protease were analyzed by enzymatic assay with Na-benzoyl-D, L-arginine 4-nitroanilide hydrochloride (BAPNA) substrate and gelatin zymography. Inhibitors and the purified protease were added simultaneously to the enzymatic assay or they were mixed and applied to zymogram gel. The activity of untreated purified protease (PP) was arbitrarily considered as 100% and its activity after heat treatment and in the presence of inhibitors are shown relative to untreated PP. Data are shown as means ± SE (n = 3–5). $^{**}P < 0.01$ and $^{****}P < 0.0001$. *E*: amino-terminal sequence of the purified protease was determined by dimethyl labeling and mass spectrometry. Amino-terminal peptide sequence of the purified protease was found to be identical to the published sequence of chicken trypsin II P29 (24).

mass spectra of five unique peptides of chicken trypsin II-P29 are shown in Supplemental Fig. S1. Chicken trypsin I-P38 (Accession Number: Q90628) having a molecular weight of 26.1 kDa at levels ~100-fold lower ($7.91e+07$) than trypsin II-P29 was also found. To further confirm the identity of the purified protease, proteases purified from poultry organic dust from two different facilities were subjected to dimethyl labeling and mass spectrometry for identification of amino-terminus. Comparison of the amino acid sequence of the dimethyl-modified amino-terminal peptide with the published sequence of chicken trypsin II-P29 (24) showed that the peptide sequence was identical to the amino-terminal sequence of chicken trypsin II-P29 confirming the identity of the purified protein as chicken trypsin II-P29 (Fig. 1*E*).

Purified Protease Induces Inflammatory Mediator Levels in a Dose-Dependent Manner in Airway Epithelial Cells and THP-1 Macrophages

In exploratory studies, the effects of various concentrations of the purified protease (0.016–0.2 μg/mL) on the induction of pro IL-1β, ICAM-1, IL-6, IL-8, and TNF protein levels in Beas2B and NHBE epithelial cells and in THP-1 macrophages were investigated. It was found that the purified protease increased pro IL-1β, ICAM-1, IL-6, and IL-8 protein levels in a concentration-dependent manner (Supplemental Fig. S2). At a concentration of 0.04 μg/mL, the purified protease induced pro IL-1β, ICAM-1, IL-6, and IL-8 in Beas2B cells (Fig. 2, *A–E*), pro IL-1β and ICAM-1 in NHBE epithelial cells (Fig. 2, *A–E*), and pro IL-1β, ICAM-1, IL-8, and TNF in THP-1 macrophages (Fig. 2,



A–F). Air-liquid interface (ALI) cultures of Beas2B cells when treated with increasing concentrations of purified protease (0.012–0.12 μg/cm^2) showed an increasing trend in pro IL-1β, IL-6, and IL-8 but not ICAM-1 protein levels (Supplemental Fig. S3). At a concentration of 0.12 μg/cm^2, the purified protease significantly increased pro IL-1β and secreted IL-8 levels in apical chamber, but not IL-8 levels in basolateral chamber and IL-6 levels in apical and basolateral chamber (Fig. 2, *G–M*). The effects of the purified protease on the induction of inflammatory mediators in Beas2B cells were studied in detail to understand the underlying mechanisms. Purified protease increased pro IL-1β, ICAM-1, IL-6, and IL-8 protein levels in a time-dependent manner with marked changes evident after 5-h treatment (Fig. 3, *A–E*). Inductions of pro IL-1β, ICAM-1, IL-6, and IL-8 proteins were associated with increases in corresponding mRNA levels (Fig. 3, *F–I*). These data indicated that the induction is controlled primarily at the level of mRNA expression. Beas2B cell viability was not reduced upon treatment with the purified protease (0.04 and 0.4 μg/mL) (Supplemental Fig. S4) indicating that increases in inflammatory mediators are not associated with cell toxicity.

Serine Protease Inhibitors Suppress the Induction of Inflammatory Mediators by the Purified Protease

Analysis by mass spectrometry identified the purified protease as chicken trypsin II-P29. To determine if the catalytic activity of the purified protease is required for the induction of inflammatory mediators, the effects of serine protease inhibitors, leupeptin and SBTI were determined. Serine protease inhibitors leupeptin and SBTI significantly inhibited purified protease induction of pro IL-1β, ICAM-1, and IL-8 proteins (Fig. 4, *A–E*) by inhibiting their corresponding mRNA levels (Fig. 4, *F–I*). Although leupeptin and SBTI did not significantly inhibit IL-6 protein levels (Fig. 4*D*), they inhibited IL-6 mRNA levels (Fig. 4*H*). Interestingly, inhibition of thymic stromal lymphopoietin (TSLP) mRNA induction was also found (Fig. 4*J*). Cell viability was not reduced by protease inhibitors (Supplemental Fig. S5). These results showed that the activity of the protease is required for the induction of inflammatory mediators.

Role of Cell Signaling Pathways in the Induction of Inflammatory Mediators by the Purified Protease

To understand the mechanisms mediating the induction of inflammatory mediators by the purified protease, the involvement of cellular signaling pathways was studied. The effects of the purified protease on the activation of signaling proteins and the effects of chemical inhibitors of signaling pathways on the induction of inflammatory mediators were investigated. Treatment of Beas2B cells with the purified protease induced time-dependent activation of MAPK1/3, MAPK8/9, and MAPK14 (Fig. 5*A*) as well as NF-κB and Stat-3 (Fig. 5*B*). MAPK1/3 activation occurred rapidly, peaking at 5 min and declined to basal levels by 60 min whereas MAPK8/9 activation peaked between 10 and 30 min declining to basal levels by 120 min (Fig. 5*A*). In contrast, although MAPK14 (Fig. 5*A*) and NF-κB (Fig. 5*B*) were rapidly activated, their activation was sustained up to 120 min. Stat-3 activation was not apparent until 60 min after treatment and declined by 120 min (Fig. 5*B*). The importance of cell signaling pathways in the induction of inflammatory mediators was investigated using chemical inhibitors targeting each signaling pathway. Chemical inhibitors targeting PKC, MAPK1/3 and MAPK14, and Stat-3 but not MAPK8/9 suppressed protease induction of pro IL-1β, IL-6, and IL-8 however, ICAM-1 levels were not affected (Fig. 5, *C–G*). Chemical inhibitor targeting NF-κB inhibited pro IL-1β and IL-6 but not ICAM-1 and IL-8 levels. Interestingly, MAPK8/9 inhibitor, SP600125 synergized protease increase of IL-6 protein levels (Fig. 5*F*). Chemical inhibitors did not adversely affect cell viability (Supplemental Fig. S5).

Purified Protease Increases Cellular and Mitochondrial ROS Levels

We found previously that dust-derived protease activities and increased ROS production contribute to the induction of inflammatory mediators by poultry organic dust (17). Treatment of Beas2B and NHBE cells with the purified protease significantly increased DCF fluorescence indicating enhanced generation of ROS (Fig. 6, *A–D*). Inhibition of protease activity with protease inhibitor leupeptin and antioxidant tempol significantly reduced DCF fluorescence (Fig. 6, *A–D*) indicating that the protease activity is responsible for the increased generation of ROS, and antioxidant suppresses ROS generation. Treatment with purified protease increased MitoSOX Red-derived fluorescence (Fig. 6, *E–H*) indicating increased production of mitochondrial superoxide in Beas2B and NHBE cells. Leupeptin and mitochondrial antioxidant MitoTEMPO, suppressed MitoSOX Red-derived fluorescence (Fig. 6, *E–H*) indicating that the protease activity may be responsible for the increased generation of mitochondrial ROS and mitochondria-targeted antioxidant suppresses ROS production.

Antioxidants Inhibit the Induction of Inflammatory Mediator Protein Levels by the Purified Protease

The effects of antioxidants such as DMTU and tempol were investigated to understand the role of oxidative stress in the induction of inflammatory mediators by the purified protease. DMTU and tempol reduced pro IL-1β, ICAM-1, and IL-8 protein levels while effects on inhibition of IL-6 levels were not significant (Fig. 7, *A–E*). Cell viability was not reduced by antioxidants (Supplemental Fig. S5).

Figure 2. Purified protease increases the levels of inflammatory mediator proteins in airway epithelial cells and THP-1 macrophage cells. *A–F*: Beas2B and NHBE airway epithelial and THP-1 macrophage cells were treated with the purified protease (PP) (0.04 μg/mL) for 5 h. *G–M*: Beas2B air-liquid interface (ALI) cultures were treated with the purified protease (PP) (0.12 μg/cm^2) at the apical side for 3 h. The levels of pro interleukin (IL)-1β and ICAM-1 proteins in cell lysates and the levels of IL-6, IL-8, and tumor necrosis factor (TNF) proteins in cell culture medium were determined by Western blotting and ELISA respectively. In case of samples in which IL-6 and IL-8 levels could not be detected, the lowest value obtained among the samples was assigned (0.208 pg/mL for IL-6 level in apical medium, 0.365 pg/mL for IL-6 level in basolateral medium, and 0.571 pg/mL for IL-8 level in basolateral medium). Pro IL-1β and ICAM-1 protein levels were normalized to actin and their levels in control cells were arbitrarily assigned as 1. Data shown are means ± SE (n = 5–8 for Beas2B, n = 3–5 for NHBE, n = 3–6 for THP-1 macrophages, and n = 6–12 for Beas2B ALI). Statistical significance was analyzed by two-tailed unpaired t test. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

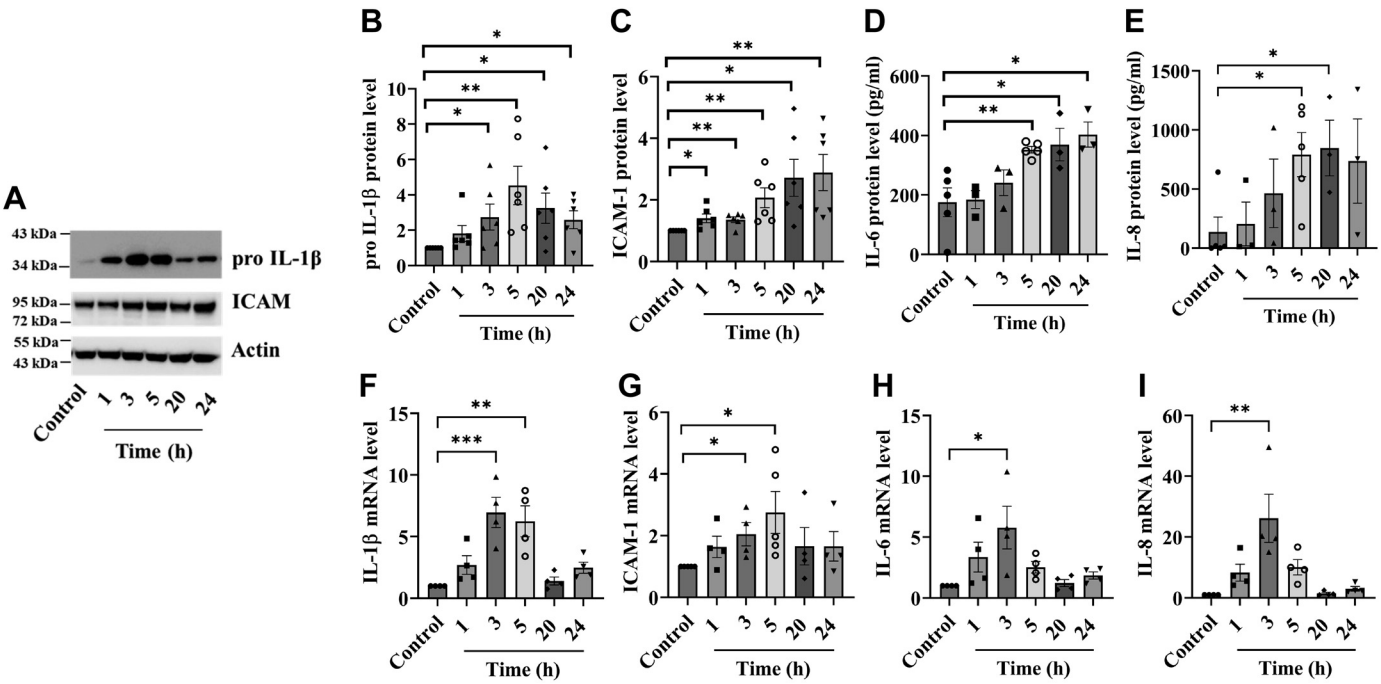


Figure 3. Purified protease increases inflammatory mediator levels in a time-dependent manner in Beas2B cells. Cells were treated with the purified protease (0.02 μg/mL) for 1, 3, 5, 20, and 24 h and protein and mRNA levels of inflammatory mediators were determined. *A–E*: levels of pro interleukin (IL)-1β and ICAM-1 proteins in cell lysates and IL-6 and IL-8 proteins in cell culture medium were determined by Western blotting and ELISA, respectively. Pro IL-1β and ICAM-1 levels were normalized to actin levels and their levels in control cells were arbitrarily assigned as 1. Representative Western blots are shown. Data shown are means ± SE (n = 3–6). *F–I:* IL-1β, ICAM-1, IL-6, and IL-8 mRNA levels were determined by real-time qRT-PCR and normalized to actin mRNA levels. mRNA levels in control cells were arbitrarily assigned as 1. Data shown are means ± SE (n = 4 or 5) and statistical significance was analyzed by two-tailed unpaired t test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Purified Protease Activates PARs

Protease-activated receptors (PARs) F2R, F2RL1, F2RL2, and F2RL3 are a family of G protein-coupled receptors that are activated via cleavage of specific N-terminal peptides by endogenous and exogenous proteases leading to cell signaling events controlling the regulation of gene expression and other cellular functions (25). Increase in intracellular calcium levels is associated with the activation of PARs (26). Beas2B cells have been reported previously to express F2R, F2RL1-L3 (27). The effects of the purified protease on changes in the intracellular calcium levels in Beas2B cells were investigated using cell-permeable calcium-sensitive fluorescent dye Fluo-4 AM. Treatment of cells with the purified protease rapidly increased cellular fluorescence, indicative of increased intracellular calcium levels. Increase in fluorescence was suppressed by leupeptin indicating possible activation of PARs by the protease (Fig. 8, *A* and *B*).

To identify PARs activated by the purified protease, cross-desensitization experiments were performed. Beas2B cells were first treated with F2R agonist peptide (TFLLRN) to desensitize F2R receptors and then exposed to the purified protease. After desensitization of the F2R receptor, the purified protease elicited an increase in intracellular calcium levels (Fig. 8*C*) whereas prior treatment with the purified protease resulted in a much smaller increase of intracellular calcium levels by the F2R agonist peptide (Fig. 8*D*) indicating that F2RL1, F2RL2, and/or F2RL3 are activated by the purified protease. Similarly, desensitization experiments with F2RL1 agonist peptide (SLIGRL) and purified protease were performed. Results showed that the F2RL1 agonist peptide rapidly increased intracellular calcium levels that slowly decreased over time and treatment with purified protease further increased calcium levels that could be due to the activation of F2R, F2RL2, and/or F2RL3 (Fig. 8*E*). Desensitization with purified protease followed by treatment with F2RL1 agonist peptide produced a small increase in intracellular calcium levels that could be indicative of incomplete desensitization and/or recycling of F2RL1 (Fig. 8*F*). We further verified purified protease activation of PARs by desensitization experiments with thrombin and trypsin. Thrombin is known to activate F2R, F2RL2, and F2RL3 (25) whereas trypsin is known to activate F2R, F2RL1, and F2RL3 with its effects on F2RL2 activation not known (25). Thrombin induced a sharp spike in intracellular calcium levels and treatment with the purified protease further increased intracellular calcium levels consistent with F2RL1 activation (Fig. 8*G*). On the other hand, when cells were treated with thrombin after treatment with purified protease, no increase in intracellular calcium levels were observed indicating activation of F2R, F2RL1, F2RL2, and F2RL3 by the purified protease (Fig. 8*H*). In agreement with these results, treatment with human trypsin or the purified protease completely abolished any increase in intracellular calcium levels by the purified protease or trypsin (Fig. 8, *I–J*). Collectively, these experiments indicated that the purified protease activates F2R, F2RL1, F2RL2, and F2RL3.

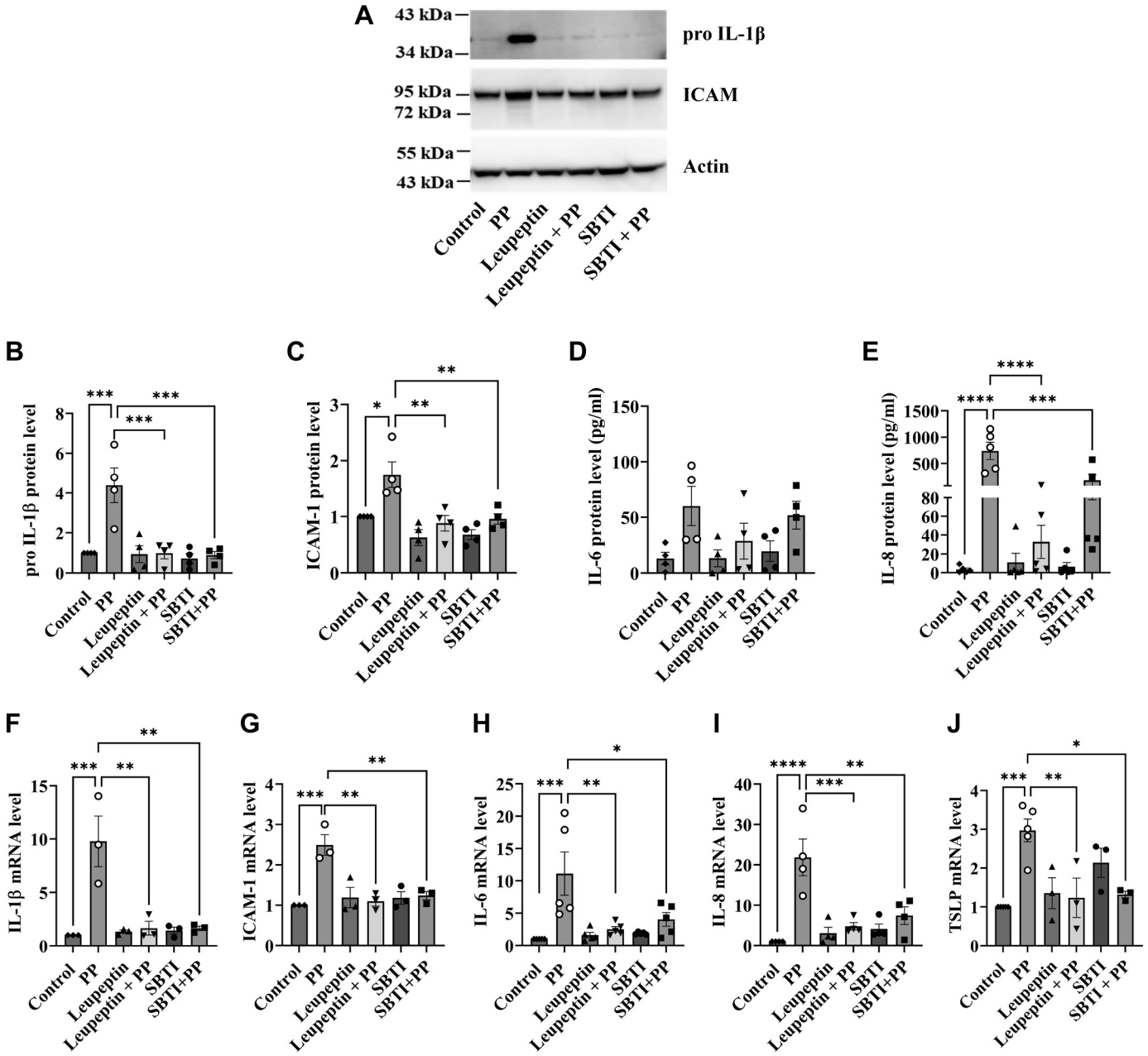


Figure 4. Serine protease inhibitors suppress purified protease induction of inflammatory mediator protein and mRNA levels in Beas2B cells. Cells were treated for 5 h with the purified protease (PP) (0.04 μg/mL), protease inhibitor alone [20 μM leupeptin or 25 μg/mL soybean trypsin inhibitor (SBTI)], or protease that had been incubated with the inhibitors for 1 h at room temperature. *A–E*: levels of pro interleukin (IL)-1β and ICAM-1 proteins in cell lysates and levels of IL-6 and IL-8 in cell culture medium were determined by Western blotting and ELISA, respectively. In case of samples in which IL-8 levels could not be detected, the lowest value obtained among the samples was assigned (0.587 pg/mL for IL-8 level). Pro IL-1β and ICAM-1 levels were normalized to actin level and their levels in control cells were arbitrarily assigned as 1. Representative Western blots are shown. Data shown are means ± SE (n = 4 or 5). *F–J*: IL-1β, ICAM-1, IL-6, IL-8, and thymic stromal lymphopoietin (TSLP) mRNA levels were determined by real-time qRT-PCR and normalized to actin mRNA levels. Their levels in control cells were arbitrarily assigned as 1. Data shown are means ± SE (n = 3–5) and statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

PARs Mediate Purified Protease Induction of Inflammatory Mediators

Analysis by mass spectrometry identified the purified protease as chicken trypsin II-P29, and intracellular calcium mobilization studies indicated activation of F2R, F2RL1, F2RL2, and F2RL3 by the purified protease. As F2R and F2RL1 are reported to be involved in the regulation of the induction of inflammatory mediators (25), we investigated their role in the induction of inflammatory mediators by siRNA-mediated knockdown experiments. Transfection of Beas2B cells with siRNAs targeting F2R and F2RL1 decreased F2R and F2RL1 mRNA levels by ~50% (Fig. 9, *A* and *B*). Treatment of transfected cells with the purified protease resulted in significant reductions in the induction of inflammatory mediators pro IL-1β, IL-6, and IL-8, but not ICAM-1 (Fig. 9, *C–G*).

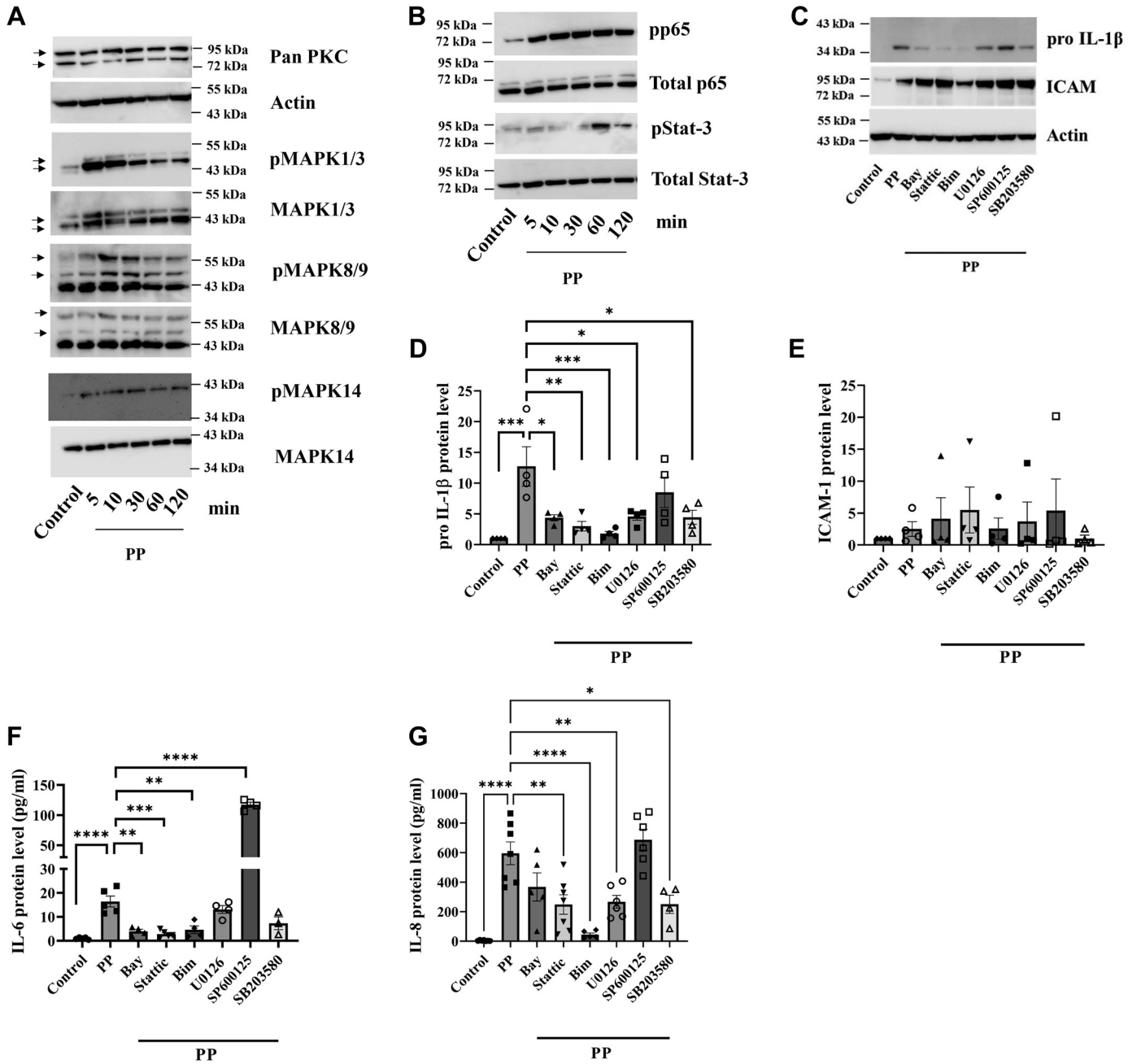


Figure 5. Purified protease activates mitogen-activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-p65 and signal transducer and activator of transcription 3 (Stat-3) and chemical inhibitors of MAPKs and NF-κB-p65 and Stat-3 suppress induction of inflammatory mediators by the purified protease. *A* and *B*: Beas2B cells were treated with the purified protease (PP) (0.02 μg/mL) for 5, 10, 30, 60, and 120 min and the levels of phosphorylated and total levels of PKC, MAPK1/3, MAPK8/9, MAPK14, NF-κB-p65, and Stat-3 were determined by Western blotting. Representative Western blots of three independent experiments are shown. *C*–*G*: Beas2B cells were treated with inhibitors targeting NF-κB (5 μM BAY 11-7082), Stat-3 (5 μM Stattic), protein kinase C (PKC) (5 μM bisindolylmaleimide), MAPK1/3 (1 μM U0126), MAPK8/9 (10 μM SP600125), and MAPK14 (10 μM SB203580) for 1 h prior to treatment with the purified protease (PP) (0.02 μg/mL) for 5 h. The levels of pro IL-1β and ICAM-1 in cell lysates and the levels of IL-6 and IL-8 in cell culture medium were determined by Western blotting and ELISA, respectively. Pro IL-1β and ICAM-1 were normalized to actin and arbitrarily assigned as 1 in control cells. Data shown are means ± SE (n = 4–7) and statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. Representative Western blots are shown.

Purified Protease Induces Lung Inflammatory Cytokines in Mice

The effects of the purified protease on lung inflammatory mediators in mice were investigated to determine if the protease induces cytokines in mouse lungs as in airway epithelial cells in vitro. The effects of intranasal instillation of the purified protease on mouse lung cytokines were determined. A single instillation of the purified protease (0.4 μg/50 μL) significantly increased BAL fluid CXCL1, IL-6, and TNF levels

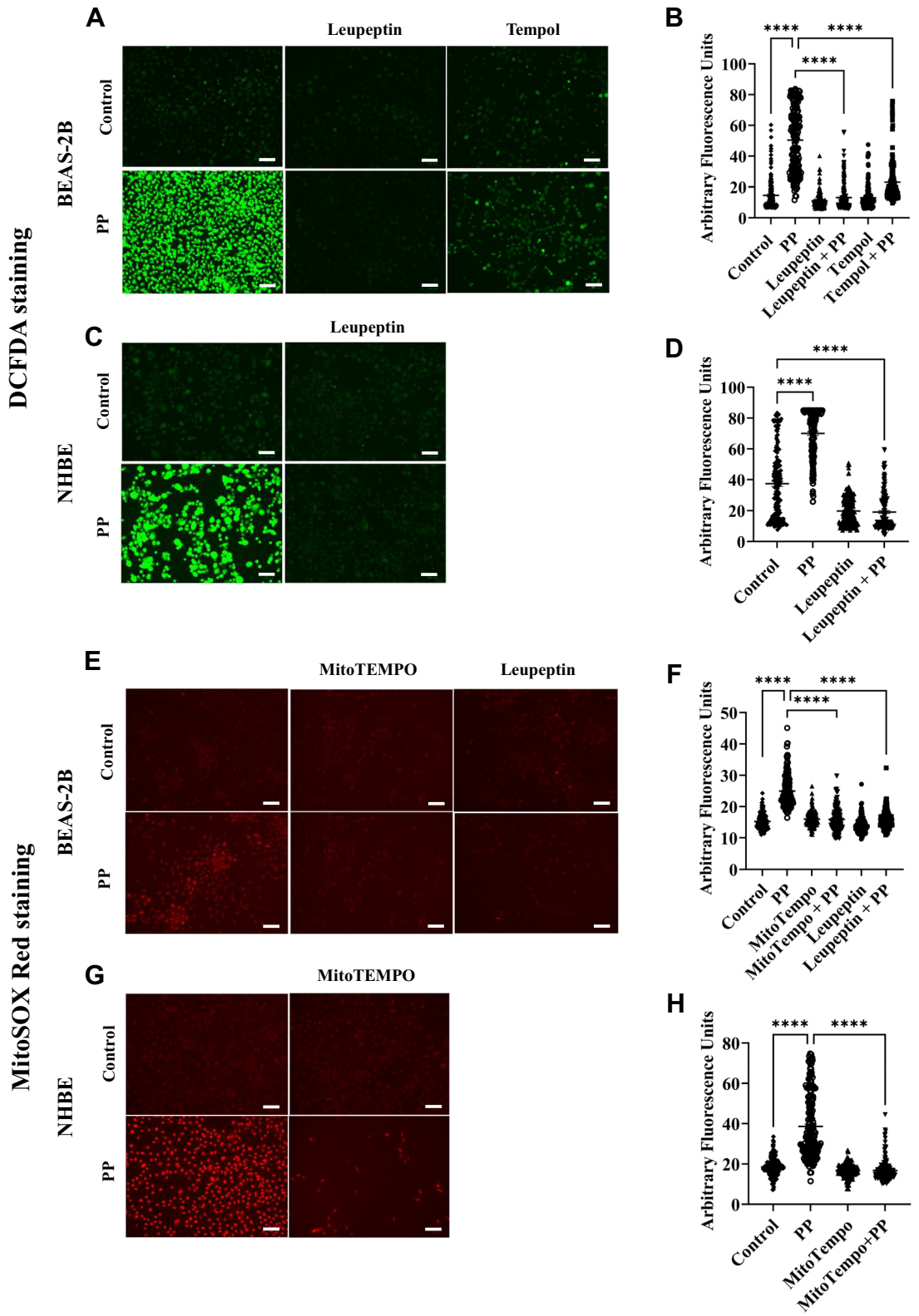


Figure 6. Purified protease increases cellular and mitochondrial reactive oxygen species (ROS) generation in Beas2B and NHBE cells. Cells were first treated for 1 h with medium alone, 2.5 mM Tempol or 10 μM mitoTEMPO and then incubated with the purified protease (PP) (0.1–0.4 μg/mL) for 30 min. To determine the effects of leupeptin, cells were treated with the purified protease that had been incubated with 20 μM leupeptin for 1 h at room temperature. Cellular and mitochondrial ROS generation were determined by staining cells with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) and MitoSOX Red, respectively. Representative images are shown for Beas2B (*A* and *E*) and NHBE (*C* and *G*) cells (scale bars = 100 μm). Fluorescence intensities were quantified in randomly selected 60 Beas2B or NHBE cells from each of three independent experiments using Image J (*B*, *D*, *F*, and *H*). Data shown are means ± SE ($n = 3$) and statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. ****$P < 0.0001$.

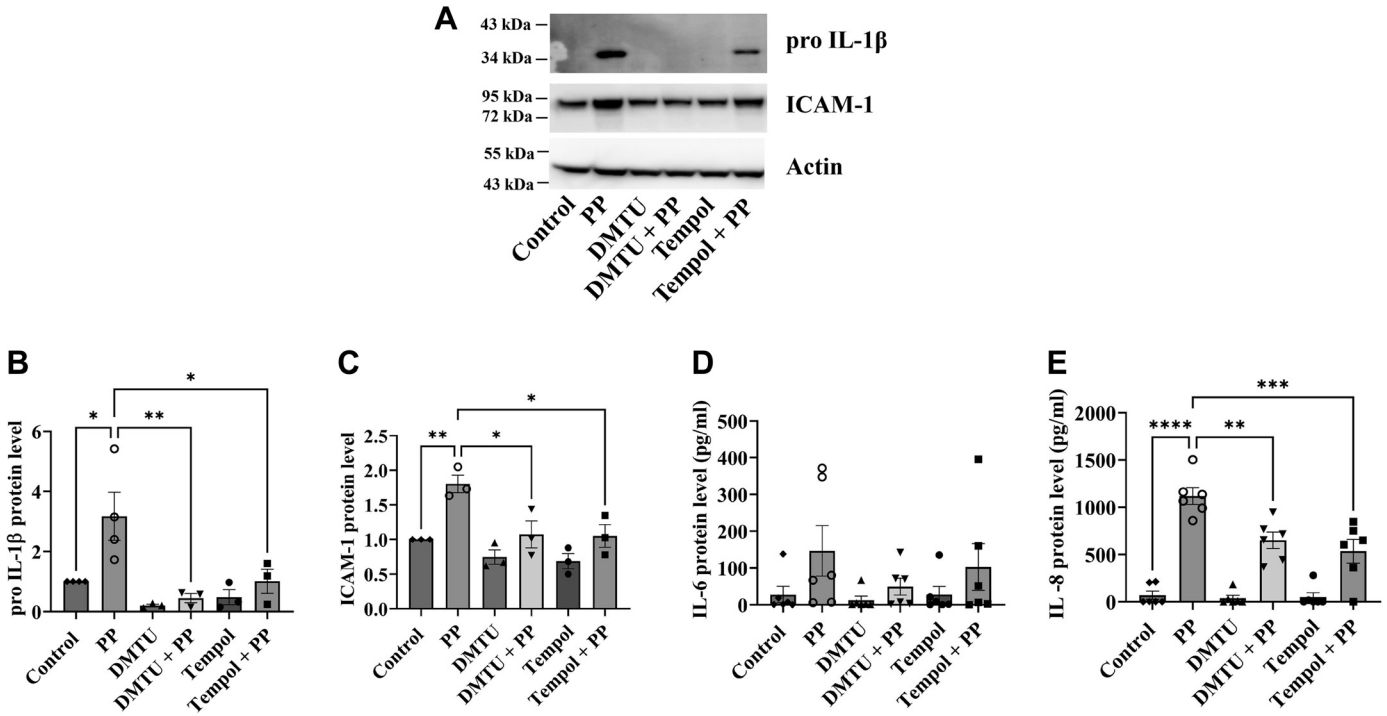


Figure 7. Antioxidants suppress purified protease induction of inflammatory mediators in Beas2B cells. Cells were treated with or without 30 mM dimethylthiourea (DMTU) and 2.5 mM tempol for 1 h before incubation with or without the purified protease (PP) (0.05 μg/mL) for 5 h. Levels of pro interleukin (IL)-1β, ICAM-1 proteins in cell lysates and levels of IL-6 and IL-8 proteins in cell culture medium were determined by Western blotting and ELISA, respectively (*A*–*E*). In case of samples in which IL-6 and IL-8 levels could not be detected, the lowest value obtained among the samples was assigned (0.374 pg/mL for IL-6 and 0.785 pg/mL for IL-8). Pro IL-1β and ICAM-1 levels were normalized to actin levels and their levels in control cells were arbitrarily assigned as 1. Representative Western blots are shown. Data shown are means ± SE (n = 3–6), and statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

after 3 h and their levels declined to near control levels after 24 h (Fig. 10, *A*–*C*). IL-1β levels were not significantly affected (Fig. 10*D*).

DISCUSSION

Organic dust is a complex mixture of materials derived from animals, feed, animal waste, bacteria, and bacterial products (1, 4). Although it is well known that organic dust exposure is associated with the development of respiratory symptoms and respiratory diseases (1, 3), the identities of the constituents and the mechanisms by which they promote respiratory dysfunction are not fully understood.

We and others have reported recently that protease activities in organic dust extracts contribute to the induction of inflammatory mediators in bronchial epithelial cells (17, 18). Bacteria, fungi, mites, animal feed, and the animals themselves could be potential sources for the proteases found in organic dust (25, 28). There appears to be little or no information on the identities of proteases found in organic dust and on the mechanisms by which they induce lung inflammation. We purified a trypsin-like protease from poultry organic dust to apparent homogeneity by benzamidine-agarose affinity chromatography. Benzamidine is a reversible competitive inhibitor of serine-proteases and has been used as a ligand for affinity purification of trypsin and other serine-proteases (23). Affinity purification resulted in a pure protein preparation as judged by SDS-PAGE and silver staining. The low yield of the purified protease (2.3%) could have resulted from lack of optimization of the purification procedure. Nevertheless, our purification procedure yielded sufficient quantities of the pure protease for characterization and functional analysis. Analysis of the purified protease by SDS-PAGE under reducing and nonreducing conditions produced a single protein band of ~26 kDa indicating that the protein is composed of a single polypeptide chain. Analysis by gelatin zymography produced a single protein band consistent with results obtained by SDS-PAGE but the size of the protein was determined to be ~17 kDa. The estimated lower size of the protein could have resulted from faster migration of the protein under conditions of gelatin zymography. Analysis of purified protease and identification of protein amino-terminus by dimethyl labeling and mass spectrometry demonstrated the purified protein as chicken trypsin II-P29. The relative molecular mass of the purified protease is similar to that of chicken trypsin II-P29 (26.6 kDa) and chicken trypsin I P48 (26.1 kDa). As proteases are abundantly expressed in the digestive tract of chickens, particularly trypsin II-P29 (29), chicken droppings, chicken discharge, and chicken dander could be potential sources of trypsin-contaminating dust in poultry farms.

Trypsin belongs to serine-protease family of proteolytic enzymes and is ubiquitously found in animals and microbes (30). It is produced in the form of inactive zymogen trypsinogen that is proteolytically cleaved to generate active trypsin (30). Chicken trypsinogen I and II share ~70% sequence similarity at the nucleotide and amino acid levels (31). Trypsins from higher animals display sequence similarities but vary

from microbial trypsins, however, all trypsins are similar structurally (30). Our understanding of proteases as enzymes solely involved in the degradation of cellular proteins has changed with the discovery that proteases play important roles in the pathogenesis of various diseases through their actions to modulate cellular functions (32). Elevated protease activity due to increased expression and/or due to an imbalance of protease-antiprotease levels is associated with the progression of lung diseases such as pulmonary fibrosis, cystic fibrosis, emphysema, and microbial infections (33–36).

As workers in agricultural and animal production operations are exposed daily to organic dust for extended periods of time, workers are at risk of exposure to proteases found in organic dust. Many of the allergens from plants, bacteria, fungi, and house dust mites (HDM) are proteases belonging to serine (trypsin, chymotrypsin, and subtilisins), aspartate (pepsin, rennin), cysteine (papain), and metalloproteinase protease families (37). A number of allergens with intrinsic protease activity cause airway inflammation leading to the development of lung diseases such as asthma, COPD, and hypersensitivity pneumonitis (32). It is known that serine proteases induce inflammation via the activation of immune cells (38). Exposure of bronchial epithelial cells to serine proteases such as trypsin results in increased secretion of pro-Th2 cytokines/proallergic cytokines that include TSLP, IL-5, and IL-33 leading to the induction of Th2 immunity (39, 40). TSLP production is induced by Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)-containing protein 2 ligands, microbes, chemicals, and proinflammatory cytokines (41). Our study showed that the purified protease increased TSLP mRNA levels in Beas2B cells suggesting a potential role for the purified protease in Th2 immunity.

Our studies found that the induction of inflammatory mediator protein levels by the purified protease was associated with increased mRNA expression. The induction was dependent on the activation of NF-κB and Stat-3 transcription factors indicating that transcriptional mechanisms control induction. We further found that oxidative stress mediates the induction of inflammatory mediators by the purified protease. We found previously that protease activities present in poultry organic dust and increased production of ROS induce inflammatory mediator levels in airway epithelial cells (10, 11, 17) suggesting that the proteases are important players in the induction of lung inflammation by poultry organic dust. It was reported previously that serine proteases increase ROS generation in normal human fetal lung fibroblasts (42). Mechanisms responsible for increased ROS generation by proteases are not known. Our recent studies showed that ROS derived from NADPH oxidases and xanthine oxidases play important roles in the induction of inflammatory mediators by poultry organic dust (43). The role of elevated levels of mitochondrial ROS produced by the purified protease on the induction of inflammatory mediators needs further investigation. Elevated ROS and nitrogen

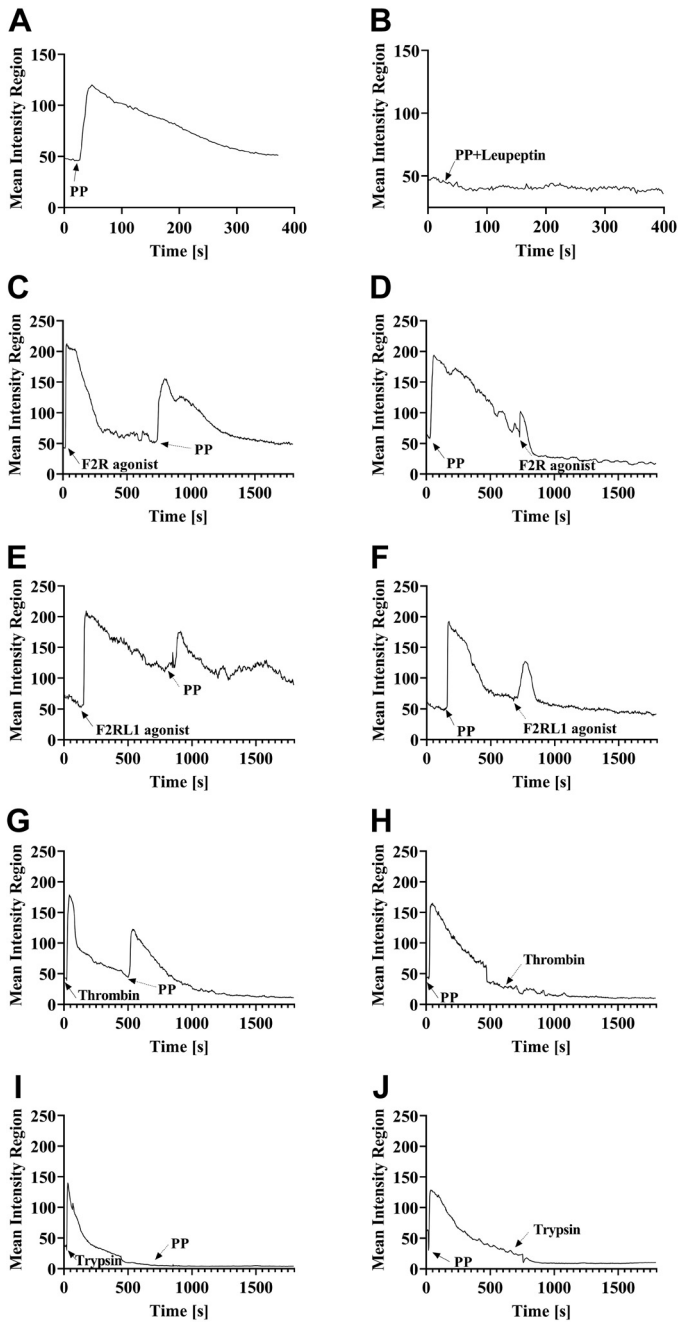


Figure 8. Purified protease (PP) increases intracellular Ca^{2+} levels in Beas2B cells via activation of protease-activated receptors (PARs). Beas2B cells were incubated with Fluo4-AM dye (4 μM) for 1 h prior to stimulation with the purified protease (PP), PP that had been incubated with 20 μM leupeptin for 1 h at 37°C, PAR agonist peptides, thrombin and human trypsin. Changes in fluorescence intensities were recorded with a confocal microscope (Zeiss) equipped with a ×20 objective (excitation/emission: 488 nm/515 nm). The following concentrations of purified protease and agonist peptides were used: purified protease (0.06 μg in 200 μL buffer B), F2R agonist peptide (50 μM), F2RL1 agonist peptide (200 μM), thrombin (10 nM), human trypsin (100 nM). Ca^{2+} levels in 3–10 cells were measured. Data from a representative experiment of three independent experiments are shown. *A*: effect of the purified protease. *B*: effect of leupeptin. *C*: effects of F2R agonist peptide followed by the purified protease. *D*: effects of the purified protease followed by F2R agonist peptide. *E*: effects of F2RL1 agonist peptide followed by the purified protease. *F*: effects of the purified protease followed by F2RL1 agonist peptide. *G*: effects of thrombin followed by the purified protease. *H*: effects of the purified protease followed by thrombin. *I*: effects of human trypsin followed by the purified protease. *J*: effects of the purified protease followed by human trypsin. Arrows show when proteases and PAR agonist peptides were added to the cells.

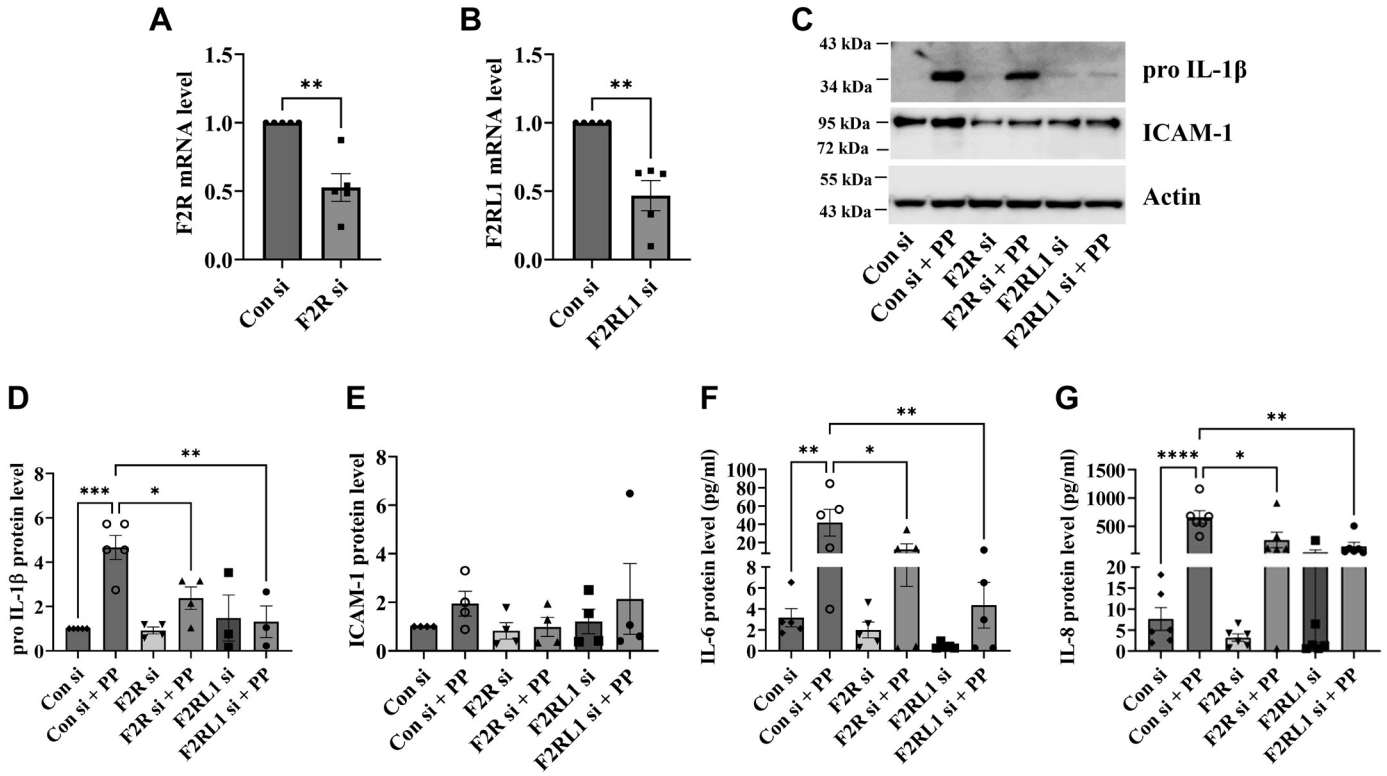


Figure 9. F2R and F2RL1 siRNA knockdown reduces purified protease induced inflammatory mediator levels in Beas2B cells. Cells were transfected with control, F2R, and F2RL1 siRNAs and grown for 65 h. Cells were treated with or without the purified protease (PP) (0.02 μg/mL) for 5 h. *A* and *B*: F2R and F2RL1 mRNA levels were determined by real-time qRT-PCR and normalized to actin mRNA levels. Data shown are means ± SE (n = 5) and two-tailed unpaired t test was used to analyze statistical significance between the two groups. *C–G*: pro IL-1β and ICAM-1 protein levels in cell lysates and IL-6 and IL-8 levels in cell culture medium were determined by Western blotting and ELISA, respectively. Representative Western blots are shown. In case of samples in which IL-6 and IL-8 levels could not be detected, the lowest value obtained among the samples was assigned (0.264 pg/mL for IL-6 and 0.581 pg/mL for IL-8). Pro IL-1β and ICAM-1 were normalized to actin and their levels in control cells were arbitrarily assigned as 1. Data shown are means ± SE (n = 3–6) and one-way analysis of variance (ANOVA) was used to analyze statistical significance between multiple groups followed by Tukey's multiple comparison test. *$P < 0.05$ and **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

radicals damage proteins, nucleic acids, and lipids to inflict cell injury and activation of stress response pathways. As a result, increased inflammation ensues leading to the development of respiratory diseases (44).

Our calcium mobilization and cross-desensitization studies indicated that the purified protease-activated F2R, F2RL1, F2RL2, and F2RL3 receptors. Although Beas2B cells express F2R, F2RL1, F2RL2, and F2RL3, F2R, F2RL1, and F2RL3 but not F2RL2 agonist peptides increased secretion of IL-6 and IL-8 in Beas2B, A549, and primary human bronchial epithelial cells (27, 45). Among the PAR agonist peptides, the potencies of induction of IL-6 and IL-8 were found to be in

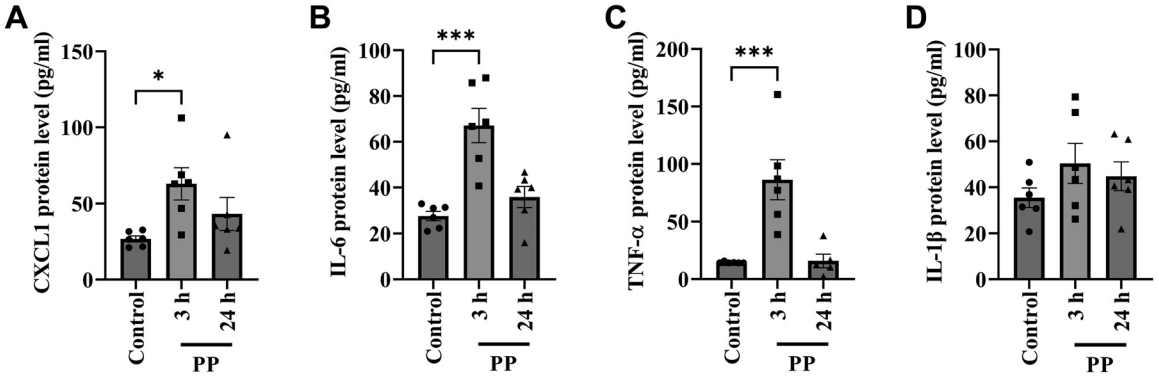


Figure 10. Purified protease (PP) increases inflammatory mediator protein levels in mice lungs. PBS (50 μL) or purified protease (PP) (0.4 μg in 50 μL PBS) was intranasally instilled into female C57BL6 mice and bronchoalveolar lavage (BAL) fluid was collected at 3 and 24 h after instillation. *A–D:* chemokine (C-X-C motif) ligand (CXCL)1, interleukin (IL)-6, tumor necrosis factor (TNF) and IL-1β levels in bronchoalveolar lavage (BAL) fluid samples were determined by ELISA. Data are shown as means ± SE (n = 6) and statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *$P < 0.05$, ***$P < 0.001$.

the following order, F2RL1 > F2RL3 > F2R (27). We and others found previously that organic dust induction of IL-6 and IL-8 in bronchial epithelial cells was sensitive to protease inhibitors and mediated by F2R and F2RL1 (17, 18). Cleavage of PARs at canonical sites by proteases results in the activation of G protein-coupled signaling pathways controlling cell responses (25). Contrary to proteases that cleave at canonical sites, certain proteases cleave PARs at other sites unmasking a new tethered ligand, leading to the activation of biased signaling pathways (46). Serine proteases such as thrombin, activated protein C (aPC), trypsin and tryptase; cysteine proteases such as calpain-1; and metalloproteases such as matrix metalloproteases (MMPs)-1, -2, -9, -12, and -13 are well studied endogenous PAR activating proteases (47). Apart from endogenous proteases, exogenous proteases derived from environmental sources such as bacteria, molds, insects, mites, pollen, and organic dust activate PARs to induce inflammatory responses. For example, cysteine protease Der p 1 from HDM *Dermatophagoides pteronyssinus* activates F2RL1 but not F2R to induce the secretion of IL-6 and IL-8 proteins in A549 lung epithelial cell line (45). LepA protease from *Pseudomonas aeruginosa* activates F2R, F2RL1, and F2RL3 (48), whereas EPa protease inactivates F2RL1 (49). Several studies have shown that PARs play major roles in the modulation of inflammation in various tissues. In the lung, F2R and F2RL1 are implicated in the development of asthma (50, 51), COPD (52, 53), and fibrosis (54).

Our studies focused on the identification and characterization of a serine protease in poultry organic dust and the understanding of its proinflammatory effects in airway epithelial and THP-1 macrophage cells. The potential contributions of other proteases that may be present in poultry organic dust were not investigated. It is not known if similar protease(s) are present in other organic dusts and if they serve as proinflammatory constituents. It would also be important to determine the levels of proteases, particularly chicken trypsin II-P29 in airborne dust in poultry farms to assess their effects on the development of respiratory symptoms and respiratory diseases. Although our studies indicated that the purified protease increased proinflammatory mediator levels in Beas2B cells under submerged and ALI conditions, future studies with pseudostratified human epithelia formed by ALI cultures of NHBE cells would be important. Nevertheless, similarities observed in our studies between airway epithelial cell cultures and mouse lungs in terms of inflammatory mediators induced by the purified protease indicate that the cell culture studies provide valuable insights into the understanding organic dust-elicited inflammatory responses.

In summary, our studies have identified chicken trypsin II-P29 as an important proinflammatory constituent of poultry organic dust. Chicken trypsin II-P29 induced IL-1β, ICAM-1, IL-6, and IL-8 by increasing their mRNA levels. Increased ROS generation and PAR activation were found to mediate the induction of inflammatory mediators via a cell signaling pathway involving PKC, MAPK1/3 and MAPK14, and NF-κB and Stat-3 transcription factors. Due to its proinflammatory properties, chicken trypsin II-P29 could contribute to the development and progression of respiratory diseases in poultry CAFO workers. Mitigating the levels of chicken trypsin II-P29 in poultry CAFO environment could serve as a useful strategy to counter chronic lung inflammation and the development of respiratory diseases in poultry agricultural workers.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S5: https://doi.org/10.6084/m9.figshare.22575169.v2.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.Kusampudi and V.B. conceived and designed research; S.Kusampudi and V.M. performed experiments; S.Kusampudi, V.M., and V.B. analyzed data; S.Kusampudi, V.M., and S.Keshava interpreted results of experiments; S.Kusampudi prepared figures; S.Kusampudi and V.B. drafted manuscript; S.Kusampudi, V.M., and V.B. edited and revised manuscript; S.Kusampudi, V.M., S.Keshava, and V.B. approved final version of manuscript.

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