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Am J Physiol Lung Cell Mol Physiol 300:L605-L614, 2011. First published 4 February 2011;
doi:10.1152/ajplung.00359.2010

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Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR₂

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Submitted 6 October 2010; accepted in final form 31 January 2011

Boitano S, Flynn AN, Sherwood CL, Schulz SM, Hoffman J, Gruzina I, Daines MO. *Alternaria alternata* serine proteases induce lung inflammation and airway epithelial cell activation via PAR₂. *Am J Physiol Lung Cell Mol Physiol* 300: L605–L614, 2011. First published February 4, 2011; doi:10.1152/ajplung.00359.2010.—Allergens are diverse proteins from mammals, birds, arthropods, plants, and fungi. Allergens associated with asthma (asthmagens) share a common protease activity that may directly impact respiratory epithelial biology and lead to symptoms of asthma. *Alternaria alternata* is a strong asthmagen in semiarid regions. We examined the impact of proteases from *A. alternata* on lung inflammation in vivo and on cleaving protease-activated receptor-2 (PAR₂) in vitro. *A. alternata* filtrate applied to the airway in nonsensitized Balb/c mice induced a protease-dependent lung inflammation. Moreover, *A. alternata* filtrate applied to human bronchial epithelial cells (16HBE14o-) induced changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i), consistent with PAR₂ activation. These effects were blocked by heat inactivation or by serine protease inhibition of *A. alternata* filtrates, and mimicked by PAR₂ specific ligands SLIGRL-NH₂ or 2-furoyl-LIGRLO-NH₂, but not the PAR₁-specific ligand TFLLR-NH₂. Desensitization of PAR₂ in 16HBE14o- cells with 2-furoyl-LIGRLO-NH₂ or trypsin prevented *A. alternata*-induced [Ca²⁺]_i changes while desensitization of PAR₁, PAR₃, and PAR₄ with thrombin had no effect on *A. alternata*-induced Ca²⁺ responses. Furthermore, the Ca²⁺ response to *A. alternata* filtrates was dependent on PAR₂ expression in stably transfected HeLa cell models. These data demonstrate that *A. alternata* proteases act through PAR₂ to induce rapid increases in human airway epithelial [Ca²⁺]_i in vitro and cell recruitment in vivo. These responses are likely critical early steps in the development of allergic asthma.

allergic asthma; *Alternaria*; inflammation; calcium; innate immunity

ALTERNARIA ALTERNATA SENSITIZATION is an important factor in the onset of childhood allergic asthma in semiarid regions (7, 11, 14, 17, 33). *A. alternata* is a complex allergen with many potentially sensitizing proteins that can directly impact epithelial cell biology. Epithelial cells provide innate immune defense against airborne environmental constituents. Many of the particulates encountered in the air we breathe are captured and removed by the mucociliary escalator. Others are engulfed by the phagocytic cells of the lung and metabolized. Certain ubiquitous environmental constituents lead to the production of a variety of cytokines and growth factors and thus contribute to airway innate immunity. These constituents include a variety of proteases from several biological

sources related to allergic asthma and include microbes, insects, and arthropods (16, 18, 35).

Allergens associated with asthma (asthmagens) contain microbe-associated molecular patterns such as lipopolysaccharide, peptidoglycan, or double-stranded DNA molecules that act on a variety of pattern recognition receptors (e.g., Toll-like receptors, Dectins, or Nod-like receptors). A second set of recognition receptors in the airway is the protease-activated receptors (PARs). The PAR family of G protein-coupled receptors (GPCR) consists of four members (PAR₁, PAR₂, PAR₃, and PAR₄) that are activated by exogenous or endogenous proteases (30, 37). Protease cleavage of the NH₂-terminus of these receptors exposes a tethered ligand that interacts with the receptors to initiate GPCR signaling pathways. PAR₂ is expressed in a variety of tissues, including the airway epithelium (38). The contribution of PAR activation to airway physiology has been the subject of many reviews (16, 18, 20, 35). However, specific roles for PAR activation in airway physiology and pathophysiology remain ill-defined.

PARs can be activated in vivo following exposure to endogenous (e.g., thrombin, tryptase, clotting factors) or exogenous (e.g., from housedust mite, *A. alternata*, cockroach) proteases. PAR₂ is unique from the other three PARs in that it is not activated by thrombin (22, 30, 34). A significant tool for the study of PARs is the use of small peptides or peptidomimetics that mimic the natural, protease-cleaved tethered ligand (26, 29, 34). These peptides provide a key advantage in studying PAR responses and subsequent cellular signaling because they directly target individual receptors without the nonspecific action of proteases. At high concentrations, peptides and peptidomimetics also can be used to desensitize cells by effectively inactivating the PAR response (23).

In this report, we provide evidence that serine-specific proteases derived from *A. alternata* induce an asthma-associated physiological response in vivo, immune cell recruitment to the lung. We further characterize *A. alternata* protease contribution to cellular signaling in human airway epithelial cells as activating PAR₂. Understanding and controlling the role(s) of allergen-associated proteases and their effect on PAR₂ will allow for insight into allergic lung diseases such as asthma.

MATERIALS AND METHODS

Materials. Eagles minimum essential medium with Earle's salts (MEM), F-12K medium, Lechner and LaVeck basal medium, Hanks' balanced saline solution (HBSS), L-glutamine, penicillin, streptomycin, geneticin, Trizol reagent, Platinum SYBR Green qPCR Super-Mix-UDG kit, Quant-iT, and the OliGreen quantification kit were purchased from Invitrogen (Carlsbad, CA). Fibronectin and type I collagen were purchased from Becton-Dickinson (Franklin Lakes,

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NJ). The iScript cDNA Synthesis kit was from Bio-Rad (Hercules, CA). Primers for real-time RT-PCR experiments were from Integrated DNA Technologies (Coralville, IA). *A. alternata* inocula to establish filtrates for in vitro experiments were from the American Type Culture Collection (ATCC, Manassas, VA). *A. alternata* filtrate used in vivo was from Greer laboratories (Lenoir, NC). Protease inhibitor cocktail (used at a final concentration of: 1.0 mM AEBSF, 0.8 μ M aprotinin, 21 μ M leupeptin, 36 μ M bestatin, 15 μ M pepstatin A, and 14 μ M E-64) was from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest biochemical quality and purchased from Sigma-Aldrich, VWR (West Chester, PA), or Fisher Scientific (Pittsburgh, PA).

A. alternata filtrate. For in vitro experiments, *A. alternata* spore suspensions (ATCC catalog no. 11680) were initially grown at 25°C on potato-dextrose agar. At day 7, 10 ml of 0.001% Tween 20 were added to the plate, and the conidia were gently dislodged using a bent glass rod. One milliliter of the resulting spore suspension ($\sim 10^6$ spores/ml) was used to inoculate 100 ml HBSS (1.3 mM CaCl₂, 5.0 mM KCl, 0.3 mM KH₂PO₄, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 137.9 mM NaCl, 0.3 mM Na₂PO₄, and 1% glucose additionally buffered with 25 mM HEPES, pH 7.4) amended with 0.12% DL-asparagine and incubated in shake culture (120 rpm) at 25°C. At day 7, fungal tissue was separated by filtering through sterile Miracloth. Filtrate was collected, standardized for protein and protease content, and then frozen for future experiments. Filtrate used in in vitro experiments contained 650 μ g/ml protein as determined with the Pierce bicinchoninic acid protein assay per the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA). Protease activity was comparable to 0.1 μ g/ml trypsin using the manufacturer's protocol supplied with the Sigma protease assay kit (catalog no. PF-0100; specific activity of trypsin not available from the supplier). If one assumes that the specific activity of the trypsin supplied in the kit were near maximal (e.g., $\sim 20,000$ BAEE U/mg), then the *A. alternata* activity would be equivalent to approximately two BAEE trypsin-like units per milliliter. For comparing different *A. alternata* preparations, the manufacturer's protocol was employed. All of the *A. alternata* protease activity was blocked by heat inactivation (30 min, 70°C) or by the serine protease inhibitor AEBSF (0.28 mM). Protease activity was not significantly altered by the cysteine protease inhibitor E-64 (10 μ M) or the aspartyl protease inhibitor pepstatin (10 μ M), as measured with this kit. Filtrate used in vivo was obtained via Greer Laboratories and dissolved in HBSS (1 mg/10 ml) with similar protease activity to that grown in culture.

Measurement of in vivo lung cell recruitment. All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Arizona. We administered 10 μ g of *A. alternata* filtrate (in 100 μ l HBSS) intranasally to 6-wk-old male Balb/c mice under brief isoflurane anesthesia. Anesthesia allowed droplets administered intranasally to be aspirated in the lungs while maintaining spontaneous respiration. *A. alternata* filtrates were administered three times over an 8-day period. Mice were euthanized on the 9th day by CO₂ inhalation 24 h after the last administration of *A. alternata* filtrate, blood was collected, and lungs were lavaged two times with HBSS by cannulation of the trachea. The collected bronchoalveolar lavage fluid (BALF) was assessed for total cell counts. Cells were cytospun to slides and stained by Diffquick. Cell differentials were determined by manual counting.

16HBE14o- cell culture. 16HBE14o- cells are a SV40 transformed human bronchial epithelial cell line (12) and were obtained through the California Pacific Medical Center Research Institute (San Francisco, CA). 16HBE14o- cells were expanded in tissue culture flasks before culture on 15-mm glass cover slips. Flasks (2 ml) and cover slips (250 μ l) were coated initially with matrix coating solution [consisting of: 88% LHC basal medium, 10% BSA (from 1 mg/ml stock), 1% bovine collagen type I (from 2.9 mg/ml stock), and 1% human fibronectin (from 1 mg/ml stock solution)] and incubated for 2 h at 37°C, after which the coating solution was removed and culture-

were allowed to dry for at least 1 h. 16HBE14o- cells were plated on the matrix-coated cultureware at a concentration of 1×10^5 cells/cm². Cells were cultured in ~ 300 μ l of control growth medium (CGM; Eagle's MEM supplemented with 10% FBS, 2 mM glutamax, penicillin, and streptomycin) at 37°C in a 5% CO₂ atmosphere. CGM was replaced every other day until the cells reached confluence (5–7 days).

Transfection of HeLa cells. HeLa cells were plated in 100-mm² tissue culture-treated petri dishes and transfected with a PAR₂-containing plasmid (PCDNA3.1 vector containing human PAR₂ with a COOH-terminus HA and an NH₂-terminus FLAG, a kind gift from Dr. Nigel Bunnett, University of California San Francisco) using FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. The plasmid was purified using the Pure Link Hi-pure Plasmid Maxiprep kit (Invitrogen) with the manufacturer's protocol. The transfection complex (6:1 ratio of FuGENE 6 to plasmid at 0.6 μ g/ μ l) was added to the cells in serum-free media without antibiotics and allowed to incubate for 6 h before replacing with regular culture media (F-12K, 10% FBS, penicillin, and streptomycin). This process was repeated 24 h later to increase transfection efficiency. Following 24 h, cells were trypsinized and transferred to T-75 flasks and allowed to grow to 95% confluence. At day 10, growth medium was replaced with selection medium (F-12K, 10% FBS, penicillin, and streptomycin, supplemented with 600 μ g/ml geneticin). At day 16, cells were trypsinized and transferred to 96-well plates in dilution series for clonal selection.

Intracellular Ca²⁺ concentration measurements. Cover slip cultures were washed with a modified HBSS and loaded for 45 min in 5 μ M fura 2-AM in HBSS. Cells were removed from fura 2-AM loading solution and placed back in HBSS for at least 20 min before Ca²⁺ imaging. Fura 2 fluorescence was observed on an Olympus IX70 microscope with a $\times 40$ oil immersion objective after alternating excitation between 340 and 380 nm by a 75-W Xenon lamp linked to a Delta Ram V illuminator (PTI) and a gel optic line. Images of emitted fluorescence above 505 nm were recorded by an ICCD camera (PTI) and simultaneously displayed on a 21-in. Vivitron color monitor. The imaging system was under software control (ImageMaster; PTI) and collected a ratio approximately every 0.6 s. Intracellular Ca²⁺ concentration ([Ca²⁺]_i) was calculated by ratiometric analysis of fura 2 fluorescence using previously published equations (13). A typical experiment consisted of 20 s of recording of cells in HBSS to determine resting [Ca²⁺]_i (typically ≤ 75 nM for 16HBE14o- or HeLa cells), followed by a 10-s wash to exchange solutions (e.g., *A. alternata* filtrate or peptidomimetic for PAR₂). Cells were monitored for an additional 2 min and 40 s. A change in [Ca²⁺]_i was considered positive if the cell increased [Ca²⁺]_i to 200 nM or more within the experimental time frame.

Real-time RT-PCR. 16HBE14o-, HeLa, and PAR₂ transfected HeLa cells were grown to confluence in T75 flasks as described above. RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and quantified using the NanoDrop ND-1000 (Thermo Fisher Scientific). cDNA was synthesized on an iCycler thermocycler (Bio-Rad) using the iScript cDNA synthesis kit according to the manufacturer's protocol and quantified using the Quant-iT OliGreen quantification kit according to the manufacturer's instructions on a TBS-380 minifluorimeter (Turner Biosystems, Sunnyvale, CA). For real-time RT-PCR, 100 ng of total cDNA/reaction were amplified with a Platinum SYBR Green qPCR Super-Mix-UDG kit in a Rotor-Gene 3000 real-time thermal cycler (Corbett Robotics, San Francisco, CA) under the following conditions: initial hold for 2 min at 50°C; hold for 2 min at 95°C; 45 cycles consisting of denature for 15 s at 94°C; anneal 30 s at 58°C; extension for 45 s at 72°C; and melted from 74 to 99°C (1°C/5 s). Primer pairs used to detect PARs and the housekeeping gene GAPDH are listed in Table 1. Individual analyses were performed in triplicate on cDNA samples.

Statistics. All statistical analyses were evaluated with GraphPad software (San Diego, CA). Multivariate comparisons were done with ANOVA with Bonferroni's multiple-comparison posttest. Pairwise

Table 1. Real-time RT-PCR primers

Gene	Primer	Primer Melt Temperature, °C	Accession No.	Primer Design Program
GAPDH	F - CGAATTTGGCTACAGCAACAGGGT	59.9	NM_002046.3	IDT-DNA Primer Quest
	R - ACTGAGGGTCTCTCTTCTCTCTTGT	60.2		
PAR ₁	F - TGGAATAAGACAGAGACCTGCCCT	59.4	NM_001992.3	IDT-DNA Primer Quest
	R - GCTGACAGGCTTGTGTTGTGAA	60.2		
PAR ₂	F - CTTTCTGTTCCAGCCTTCCTC	57.7	NM_005242.4	MacVector Software
	R - GCCCTCTTCTTTTCTTCTCTGAGT	58.3		
PAR ₃	F - TGCCATCTACCTCCTGGTGTGTTGT	60.2	NM_004101.2	IDT-DNA Primer Quest
	R - AAATCTGCAATGGCCAGGTGGTG	60.5		
PAR ₄	F - ATCTACTACTACGTGTCGGCCGAGTT	60.6	NM_003950.2	IDT-DNA Primer Quest
	R - ACTGGAGCAAAGAGAGTGGGT	60.4		

PAR, protease-activated receptor; F, forward; R, reverse.

comparisons were done with a two-tailed Student's *t*-test. A value of $P < 0.05$ was used to establish a significant difference between samples. Data in Figs. 1–8 are graphed $\pm$ SE unless otherwise noted.

RESULTS

A. alternata protease activity induces immune cell recruitment in murine airways. To determine if the asthmagen *A. alternata* had an inflammatory effect on the airway, we developed an in vivo murine model of exposure to *A. alternata* filtrates. Mice were exposed to 10 μ g *A. alternata* filtrates or control buffer (HBSS) at days 1, 4, and 8 and euthanized on day 9 (Fig. 1A). *A. alternata*-exposed mice had an average of 2.2×10^6 cells recovered from BALF ($n = 5$), a 16-fold increase over mice administered buffer (HBSS) control ($n = 5$;

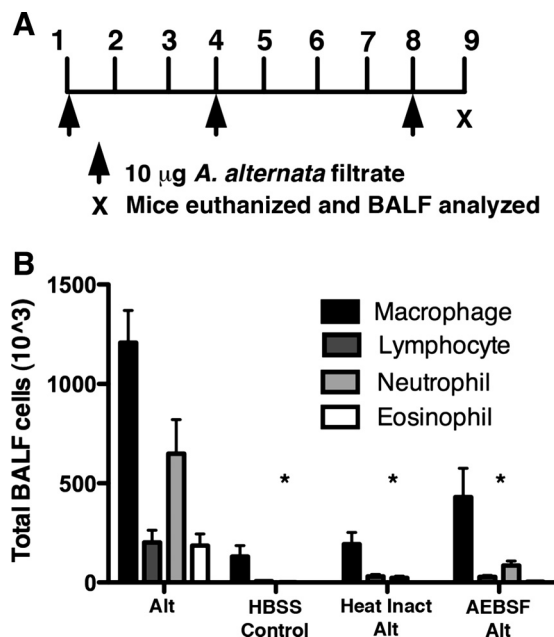


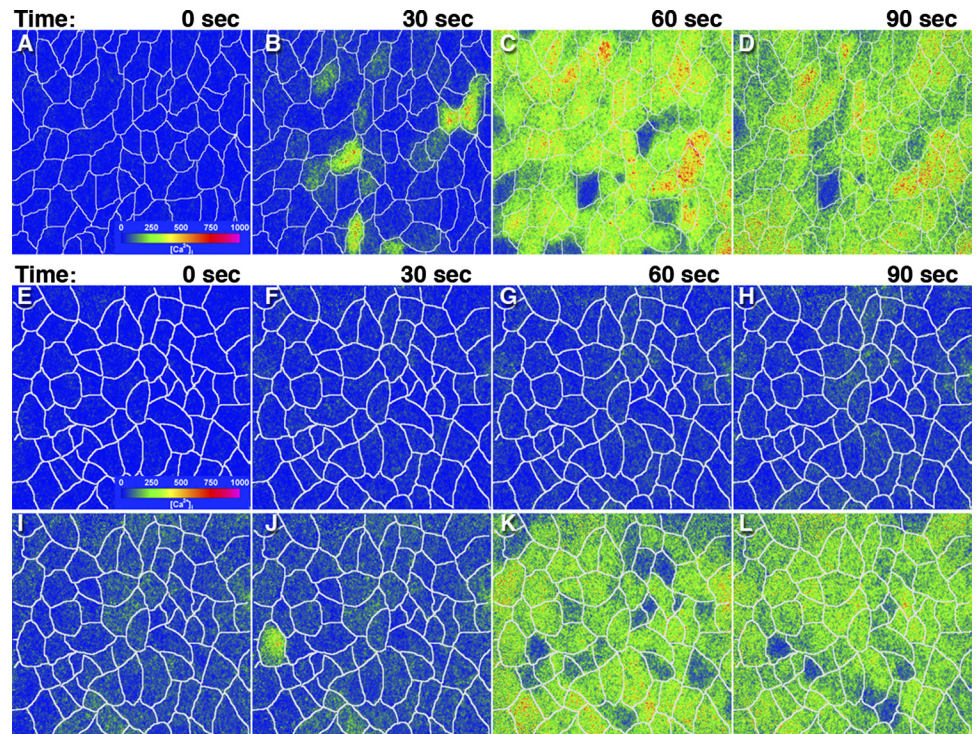
Fig. 1. *Alternaria alternata* filtrate induces lung inflammation by protease-dependent mechanisms. Adult Balb/c mice were exposed to *A. alternata* filtrate, *A. alternata* filtrate with the serine protease inhibitor AEBSF, or heat-inactivated *A. alternata* filtrate using the protocol outlined in *A. alternata* filtrate (Alt) exposure induced a significant increase in lung inflammation as shown by cell recruitment in lung bronchoalveolar lavage fluid (BALF) compared with control [Hanks' balanced salt solution (HBSS)], heat-inactivated filtrates (Heat Inact Alt), or AEBSF-treated filtrates (AEBSF Alt). *Significantly reduced cell recruitment for all cell types ($P < 0.05$) compared with control; $n = 5$ for each experiment. AEBSF alone had no effect on lung inflammation (Daines, unpublished observation).

Fig. 1B). To rule out inert contents of the filtrate (e.g., chitin), the filtrates were heated to 70°C for 30 min. Administration of heat-inactivated filtrate resulted in a significant reduction of cell recruitment (2.5×10^5 ; $n = 5$) compared with the untreated filtrate. To more closely evaluate the role for serine proteases in cell recruitment, the experiment was repeated using *A. alternata* filtrate pretreated with the serine protease specific inhibitor AEBSF (0.1 mM). Under these conditions, cell recruitment was again significantly reduced, with only 5.4×10^5 cells recovered from the BALF ($n = 5$). Mean cellular differential counts showed a statistically significant increase in lymphocytes, neutrophils, macrophages, and eosinophils in mice exposed to *A. alternata* filtrate proteases compared with mice exposed to HBSS control. There was also a significant increase in all immune cell counts in mice exposed to *A. alternata* compared with those exposed to heat-inactivated or serine protease-inhibited filtrates. These data show that serine-specific protease activity of *A. alternata* is required to develop lung inflammation as detected by cell recruitment to the airway.

A. alternata exposure results in protease-dependent $[Ca^{2+}]_i$ changes in human airway epithelial cells. Because secreted fungal proteases can increase the asthmagenic effects of *A. alternata*, we directly investigated their impact on cellular activation of respiratory epithelial cells. We exposed 16HBE14o- cells to *A. alternata* filtrate at the apical membrane to best mimic potential asthmagen-host interactions. Exposure to *A. alternata* filtrates induced a Ca^{2+} response (increased $[Ca^{2+}]_i$ to ≥ 200 nM) in 16HBE14o- cells that typically started 20–45 s following application and included $86.0 \pm 5.7\%$ ($n = 9$) of the cells within the field of view within the 180-s experiment [Fig. 2, A–D, and the Supplemental Movie (Supplemental data for this article may be found on the American Journal of Physiology: Lung Cellular and Molecular Physiology website.)]. To determine the contribution of heat-labile components to cellular activation, *A. alternata* filtrates were heated to 70°C for 30 min before application to 16HBE14o- cells. In contrast to the untreated *A. alternata* filtrates, heat-inactivated filtrate induced Ca^{2+} responses in only $1.5 \pm 1.7\%$ cells ($n = 4$; Fig. 2, E–H). Replacement of the heat-inactivated filtrate with untreated filtrate resulted in a full Ca^{2+} response ($n = 3$; Fig. 2, I–L).

Because heat can alter many components of *A. alternata*, we specifically evaluated the contribution of proteases by repeating these experiments following *A. alternata* protease inactivation. *A. alternata* filtrate was first preincubated with a broad-spectrum protease inhibitor cocktail for 5 min and applied to

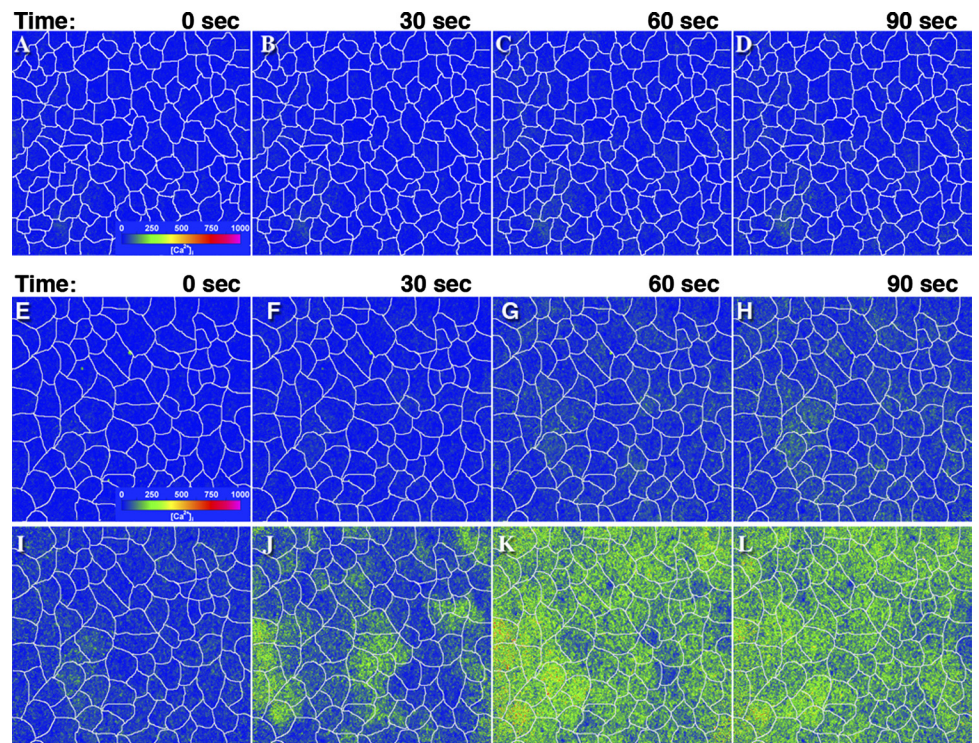
Fig. 2. *A. alternata* filtrate induces increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in human airway epithelial cells that is inactivated by heat treatment. Panels from left to right display color maps of $[\text{Ca}^{2+}]_i$ of 16HBE14o- cells over time in response to bath application of *A. alternata* filtrate. A–D: within 30 s of filtrate application, 16HBE14o- cells within the field displayed an increase in $[\text{Ca}^{2+}]_i$; by 90 s, most of the cells in the field have responded. E–H: when the experiment was repeated with heat-inactivated filtrate (70°C for 30 min), no change in $[\text{Ca}^{2+}]_i$ was observed throughout the 3-min experiment. I–L: the same cells washed with untreated filtrate increased $[\text{Ca}^{2+}]_i$ similar to that observed in control experiments. Panels detail representative experiments from an $n = 4$ for each experiment. Application of filtrate is given as time “0” with all times (s) listed at the top; for I–L, time 0 reflects the exchange of fresh filtrate in a continuous experiment with E–H. White lines depict cell borders. A color bar indicating approximate $[\text{Ca}^{2+}]_i$ is given in the bottom right of the first panel for each wash.



16HBE14o- cells. Protease-treated filtrate induced $[\text{Ca}^{2+}]_i$ responses in only $2.2 \pm 2.1\%$ ($n = 5$) of 16HBE14o- cells (Fig. 3, A–D). To further evaluate the type of proteases that were activating the 16HBE14o- cells, we preincubated *A. alternata* filtrate with protease inhibitors that reduced *A. alternata* protease activity (see MATERIALS AND METHODS). We first used the serine protease inhibitor AEBSF (0.28 mM). Pretreatment of *A.*

alternata with AEBSF prevented Ca^{2+} responses; only $3.1 \pm 1.6\%$ ($n = 4$) of the cells displayed $[\text{Ca}^{2+}]_i$ above 200 nM (Fig. 3, E–H). As observed in the heat-inactivated experiments, replacement of the AEBSF-treated filtrate with untreated filtrate resulted in a full Ca^{2+} response of the 16HBE14o- cells ($n = 3$; Fig. 3, I–L, and the Supplemental Movie). In contrast to the AEBSF results, pretreatment of *A. alternata* filtrates with

Fig. 3. *A. alternata*-induced $[\text{Ca}^{2+}]_i$ changes in human airway epithelial cells are protease-dependent. Panels from left to right display color maps of $[\text{Ca}^{2+}]_i$ of 16HBE14o- cells over time in response to bath application of *A. alternata* filtrate with and without protease inhibition. A–D: *A. alternata* filtrate incubated with a protease inhibitor cocktail does not induce $[\text{Ca}^{2+}]_i$ changes in 16HBE14o- cells. E–H: *A. alternata* filtrate incubated with the serine-specific protease inhibitor AEBSF (0.42 mM) does not induce $[\text{Ca}^{2+}]_i$ changes in 16HBE14o- cells. I–L: wash of the same field with untreated *A. alternata* filtrate results in a $[\text{Ca}^{2+}]_i$ increase similar to control. Panels detail representative experiments from an $n \geq 3$. Time (s), white lines, and $[\text{Ca}^{2+}]_i$ color bar are as detailed in Fig. 2.



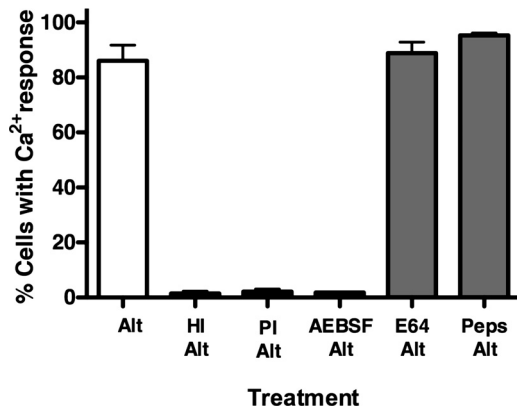


Fig. 4. Comparison of *A. alternata* and protease-activated receptor-2 (PAR₂) agonist treatments on [Ca²⁺]_i changes in human airway epithelial cells. In all experiments, cells underwent a bath exchange of HBSS to treatment solution at 20–30 s with constant monitoring of [Ca²⁺]_i over 3 min using digital imaging microscopy. The %Ca²⁺ response, or the % of cells within the field of view that increased resting [Ca²⁺]_i (~75 nM) to ≥200 nM during the experiment are plotted for each treatment. *A. alternata* filtrate (Alt) was resulted in activation of 86.0 ± 5.7% of the cells. Heat-inactivated filtrate (HI Alt; 1.49 ± 1.7%), pretreatment of filtrate with nonspecific protease inhibitors (PI Alt; 2.16 ± 2.1%), or pretreatment with the serine-specific protease inhibitor AEBSF (AEBSF Alt; 0.28 mM; 3.1 ± 1.6%) all limited *A. alternata*-induced changes of [Ca²⁺]_i within the field of view. Pretreatment of *A. alternata* filtrate with the cysteine protease inhibitor E-64 (E64 Alt; 10 μM) or the aspartyl protease inhibitor pepstatin (Peps Alt; 10 μM) did not significantly alter the Ca²⁺ response. Taken together, experiments are consistent with *A. alternata* protease activation of 16HBE14o- cells that acts on PAR₂ to induce rapid increases of [Ca²⁺]_i. Columns are graphed ± SE for each time point; *n* ≥ 4 for each treatment.

the cysteine protease inhibitor E-64 (10 μM; 88.8 ± 4.0%, *n* = 4) or pepstatin (10 μM; 95.3 ± 0.9%, *n* = 3) had no measurable effect on *A. alternata*-induced Ca²⁺ response. A summary graph with quantification of Ca²⁺ responses of 16HBE14o-cells by various *A. alternata* treatments is shown in Fig. 4. These data demonstrate that serine-specific protease(s) are responsible for *A. alternata* filtrate-induced Ca²⁺ signaling in human airway epithelial cells.

A role for PAR₂ in *A. alternata*-induced Ca²⁺ signaling in human airway epithelial cells. Proteases can induce changes in [Ca²⁺]_i in cells by cleaving and activating any of the PARs

(30). PARs have been shown to be expressed in 16HBE14o-cells by RT-PCR (1). We first directly tested to see if PAR₂ activation in 16HBE14o- cells resulted in Ca²⁺ signaling using the PAR₂-specific peptide agonist SLIGRL-NH₂ (29) and the potent peptidomimetic 2-furoyl-LIGRLO-NH₂ (21, 26). The SLIGRL-NH₂ peptide induced Ca²⁺ signaling in 88.2 ± 7.1% over a 3-min experiment (*n* = 3; Fig. 5, A–D). The 2-furoyl-LIGRLO-NH₂ peptidomimetic was similarly effective (93.6 ± 8.7%; *n* = 7) but at a much lower concentration (2.5 μM; Fig. 5, E–H). These data demonstrate that direct activation of PAR₂ can lead to robust Ca²⁺ signaling.

To experimentally evaluate potential contributions of PAR₁, PAR₃, or PAR₄ in 16HBE14o- Ca²⁺ activation, we used a desensitization protocol (23, 26) and monitored changes in [Ca²⁺]_i following addition of thrombin (Fig. 6). Thrombin is a potent protease activator of PAR₁, PAR₃, and PAR₄ but does not activate PAR₂ (37). Multiple additions of thrombin effectively desensitize PAR₁, PAR₃, and PAR₄, but does not affect PAR₂ responses in epithelial cells. Consistent with PAR₂ as the dominant PAR in 16HBE14o- cells, thrombin (100 nM) application resulted in a modest Ca²⁺ response (15.5 ± 3.0%, *n* = 16). A second addition of thrombin resulted in a reduced Ca²⁺ response (5.5 ± 1.9%). A third wash with thrombin resulted in no Ca²⁺ response (*n* = 5), indicating full desensitization of PAR₁, PAR₃, and PAR₄. However, when 16HBE14o- cells desensitized with three washes of 100 nM thrombin were followed with 2.5 μM 2-furoyl-LIGRLO-NH₂ (*n* = 5) or *A. alternata* filtrate (*n* = 5), they responded with full Ca²⁺ activation (99.1 ± 0.5 and 94.8 ± 1.7%, respectively; Fig. 6). To confirm the prominent role for PAR₂ in protease activation of 16HBE14o- cells, we used real-time RT-PCR to evaluate the relative mRNA expression of PAR₁, PAR₃, and PAR₄ compared with PAR₂ in 16HBE14o- cells. Of the four PARs, PAR₂ displayed the highest level of expression with PAR₁ also showing significant, albeit much reduced, expression. PAR₃ and PAR₄ displayed limited expression (data not shown). These data are consistent with a PAR₂ dominant protease response to *A. alternata* filtrates.

We used a second desensitization protocol (23, 26) to further evaluate if functional PAR₂ was required for *A. alternata*-induced Ca²⁺ changes (Fig. 7). First, 16HBE14o- cells were

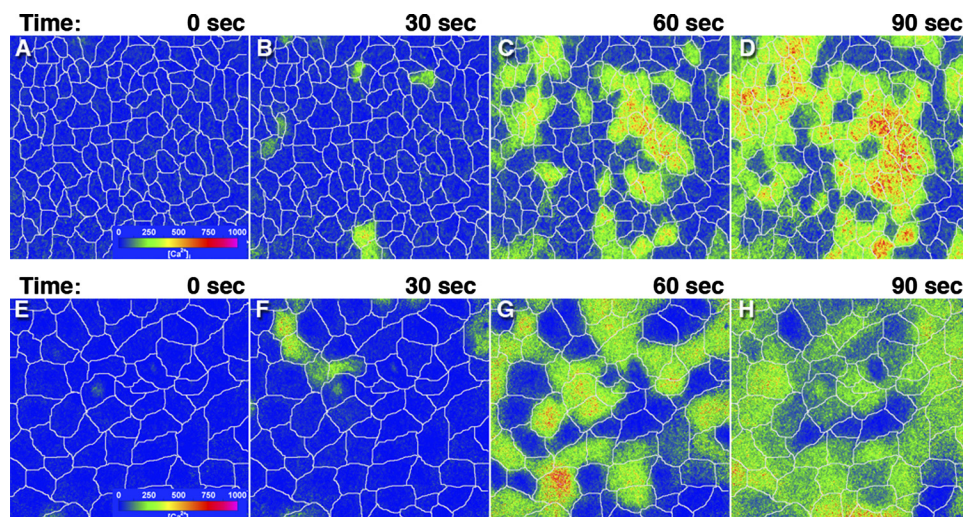


Fig. 5. PAR₂ agonists induce [Ca²⁺]_i changes in airway epithelial cells. Panels from left to right display color maps of [Ca²⁺]_i of 16HBE14o- cells over time in response to application of PAR₂ specific agonists. A–D: the peptide agonist SLIGRL-NH₂ (80 μM) increases [Ca²⁺]_i in 16HBE14o-cells within 30 s; by 90 s, most cells in the field of view have responded. E–H: the peptidomimetic agonist 2-furoyl-LIGRLO-NH₂ (2.5 μM) displays similar [Ca²⁺]_i increases in 16HBE14o- cells. Peptide and peptidomimetic agonist concentrations were chosen to display full Ca²⁺ response and are representative from an *n* ≥ 3. Application of filtrate is given as time 0 with all times (s) listed at the top. White lines depict cell borders. A color bar indicating approximate [Ca²⁺]_i is given in the bottom right of the first panel for each experiment.

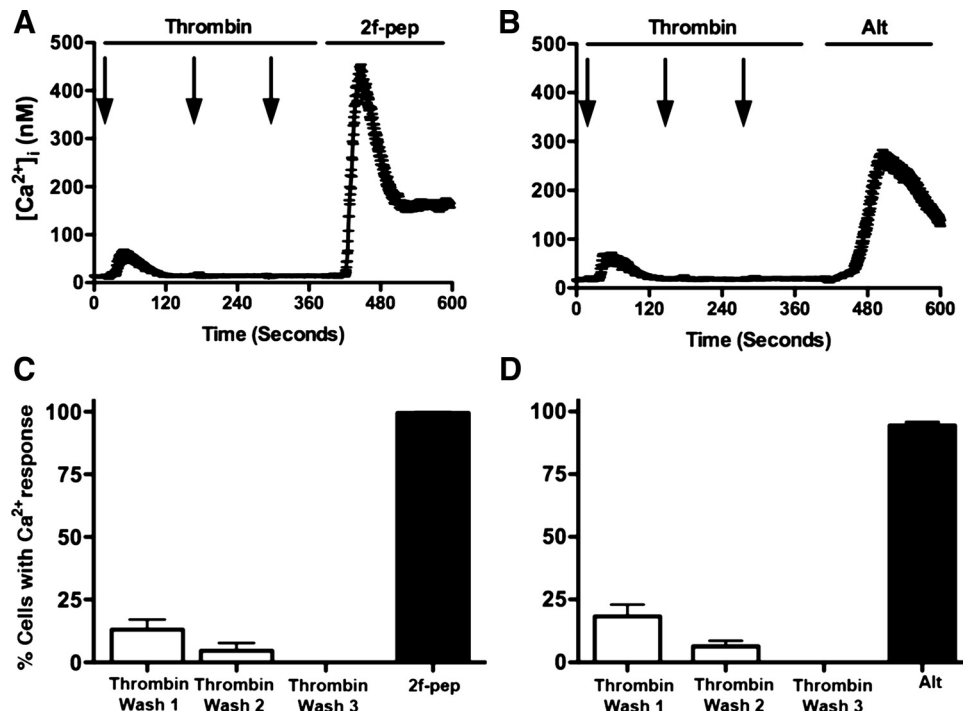


Fig. 6. Desensitization of PAR₁, PAR₃, and PAR₄ with thrombin has no effect on *A. alternata*-induced Ca^{2+} changes in human airway epithelial cells. *A* and *B*: average $[Ca^{2+}]_i$ of 80–110 cells in a field of view are plotted over time. Application of 100 nM thrombin to 16HBE14o- cells resulted in a minimal increase in $[Ca^{2+}]_i$. Second and third applications of thrombin caused little $[Ca^{2+}]_i$ change. Application of 2.5 μ M of the PAR₂ specific agonist 2-furoyl-LIGRLO-NH₂ (2f-pep; *A*) or *A. alternata* filtrate (Alt; *B*) resulted in rapid and large $[Ca^{2+}]_i$ changes throughout the cell culture. *C* and *D*: quantification of thrombin desensitization experiments. The first treatment with thrombin resulted in a small Ca^{2+} response ($20.7 \pm 6.2\%$ in *C* and $16.0 \pm 5.6\%$ in *D*) consistent with low expression of PAR₁, PAR₃, and PAR₄. Following recovery to baseline $[Ca^{2+}]_i$, a second treatment with thrombin resulted in a smaller Ca^{2+} response (8.7 ± 2.3 and $7.4 \pm 4.5\%$), whereas a third application did not induce a $[Ca^{2+}]_i$ change, consistent with desensitization of PAR₁, PAR₃, and PAR₄. Subsequent application of 2.5 μ M 2-furoyl-LIGRLO-NH₂ or *A. alternata* filtrate resulted in a full Ca^{2+} response (99.8 ± 0.2 and $94.3 \pm 1.9\%$, respectively). These data are consistent with PAR₂ as the protease target of *A. alternata* proteases. Traces in *A* and *B* represent >85 cells in a single experiment and are graphed $\pm$ SE; $n = 5$ for each experimental protocol.

exposed to high concentrations of trypsin to effectively desensitize PAR₂. Trypsin addition caused an immediate Ca^{2+} response in 100% of the 16HBE14o- cells. Following recovery to baseline $[Ca^{2+}]_i$ and a second addition of trypsin, <1% of the cells displayed a Ca^{2+} response. When cells were exposed to *A. alternata* following a desensitization, there was no increase in $[Ca^{2+}]_i$ ($n = 4$). However, the addition of ATP, which acts on purinergic receptors (2), caused a rapid increase in $[Ca^{2+}]_i$ in $76.9 \pm 15.6\%$ of cells, indicating that cells were still attached and capable of responding to GPCR ligands (Fig. 7, *A* and *C*). The ATP response was comparable to 16HBE14o-cells exposed to *A. alternata* filtrate without trypsin pretreatment (data not shown). To more directly examine the role of PAR₂ in *A. alternata*-induced Ca^{2+} signaling, we repeated the desensitization protocol using a high concentration (100 μ M) of 2-furoyl-LIGRLO-NH₂ ($n = 4$; Fig. 7, *B* and *D*). Similar to the trypsin experiments, pretreatment with 2-furoyl-LIGRLO-NH₂, where 100% of 16HBE14o- cells displayed a Ca^{2+} response, limited the *A. alternata*-induced Ca^{2+} response to <1% of the cells without affecting the ATP response ($92.0 \pm 1.8\%$). As shown, *A. alternata*-induced cellular activation is abrogated by pretreatment with either trypsin or PAR₂ agonists. Taken together, these data suggest *A. alternata* protease(s) act on PAR₂ to initiate changes in $[Ca^{2+}]_i$ in human airway epithelial cells.

To examine if PAR₂ is necessary for the cellular responses to *A. alternata*, we used HeLa cells stably transfected with

human PAR₂ as an epithelial cell model. HeLa cells displayed a distinctly different PAR profile compared with 16HBE14o-, with PAR₁ as the most abundantly expressed PAR followed by measurable expression of PAR₂ (Fig. 8*A*). This fundamental change in PAR expression is reflected in Ca^{2+} responses to PAR₁ [TFLLR-NH₂; (23)] and PAR₂-specific agonists (Fig. 8*B*). Addition of 50 μ M TFLLR-NH₂ resulted in a limited Ca^{2+} response in 16HBE14o- cells ($0.7 \pm 0.4\%$), whereas 80 μ M SLIGRL-NH₂ or 2.5 μ M 2-furoyl-LIGRLO-NH₂ both resulted in robust Ca^{2+} responses (88.2 ± 4.1 and $93.6 \pm 3.3\%$, respectively; also see Fig. 5). In contrast, addition of 25 μ M TFLLR-NH₂ induced Ca^{2+} responses in 66.8 $\pm$ 6.4% of the HeLa cells, whereas 5 μ M of 2-furoyl-LIGRLO-NH₂ induced a minimal Ca^{2+} response ($2.0 \pm 1.4\%$). Because HeLa cells express a limited amount of endogenous PAR₂, we have established a number of clones stably transfected with varying amounts of human PAR₂, as measured by real-time RT-PCR (Fig. 8*C*). To determine the effect of PAR₂ expression on *A. alternata* activation, we repeated the $[Ca^{2+}]_i$ response experiments with *A. alternata* filtrate (Fig. 8*D*). Only $2.0 \pm 1.4\%$ ($n = 5$) of the nontransfected HeLa cells were activated by *A. alternata* filtrate. In the HeLa transfectant with the low PAR₂ mRNA expression (clone 8), a significant increase in response to *A. alternata* filtrate was observed ($17.3 \pm 6.8\%$; $n = 3$). A medium expression clone (clone 7) again displayed a significant increase in response ($30.3 \pm 10.9\%$; $n = 3$), whereas the high-expression HeLa transfectant (clone 4B) displayed the

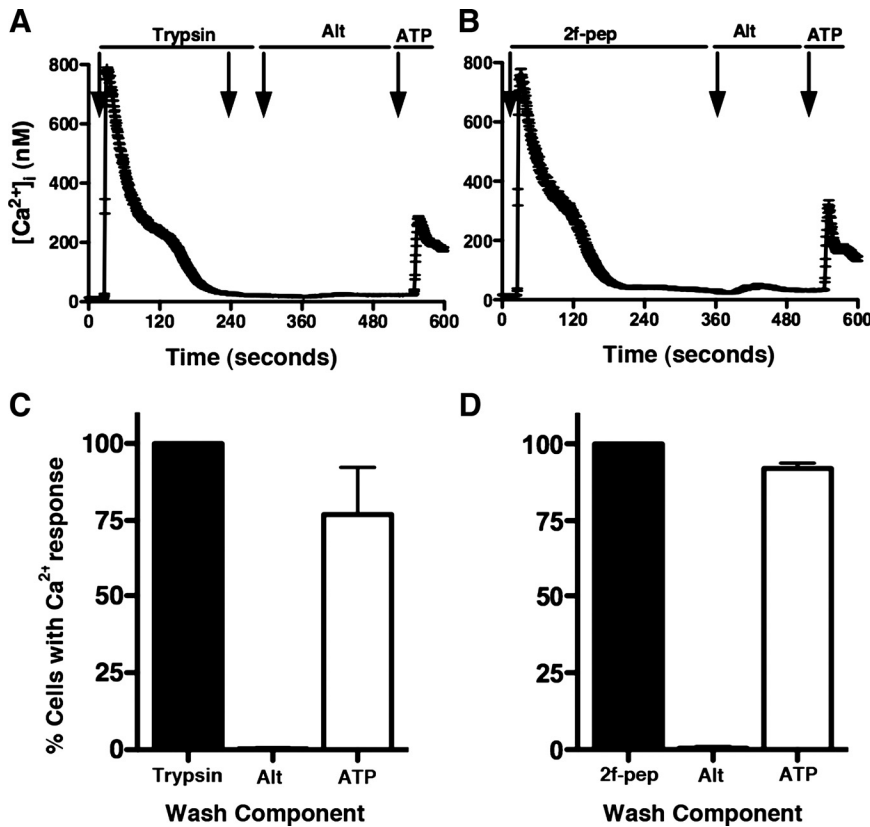


Fig. 7. Desensitization of PAR₂ eliminates *A. alternata*-induced Ca²⁺ changes in human airway epithelial cells. Average [Ca²⁺]_i of 80–110 cells in a field of view are plotted over time. **A:** application of 100 μM trypsin to 16HBE14o- cells resulted in a rapid and large increase in [Ca²⁺]_i that recovered to baseline in 3–4 min. A second application of trypsin did not induce further changes in [Ca²⁺]_i. Application of *A. alternata* filtrate (Alt) also resulted in no change in [Ca²⁺]_i. However, application of ATP resulted in a full [Ca²⁺]_i response. **B:** application of the PAR₂ selective peptidomimetic agonist 2-furoyl-LIGRLO-NH₂ (100 μM; 2f-pep) resulted in a rapid increase in [Ca²⁺]_i. Subsequent addition of *A. alternata* filtrate did not result in an increase in [Ca²⁺]_i, whereas application of ATP resulted in increased [Ca²⁺]_i. **C:** quantification of PAR₂ desensitization by trypsin. The first treatment with trypsin resulted in a full Ca²⁺ response (100% in all cultures), whereas a second application of trypsin (no Ca²⁺ response) or subsequent application of *A. alternata* filtrate (0.4 ± 0.4%) resulted in negligible Ca²⁺ responses. However, application of ATP, an agonist that works independently of PAR₂ to raise [Ca²⁺]_i, demonstrated a full Ca²⁺ response (76.9 ± 15.3%). **D:** quantification of PAR₂ desensitization by 2-furoyl-LIGRLO-NH₂ (2f-pep). The first treatment with 2-furoyl-LIGRLO-NH₂ resulted in a full Ca²⁺ response (100% in all cultures). Subsequent application of *A. alternata* filtrate (0.52 ± 0.52%) resulted in negligible Ca²⁺ responses. As seen in the trypsin desensitization, application of ATP resulted in a full Ca²⁺ response (92.0 ± 1.8%). These data are consistent with PAR₂ as the protease target of *A. alternata* proteases. Traces in **A** and **B** represent ≥85 cells from a single experiment and are graphed ± SE at each time point; *n* = 3 for each experimental protocol.

highest response (55.6 ± 9.9%; *n* = 6). Importantly, response to *A. alternata* filtrate was dependent on the amount of PAR₂ expressed in each clone, further emphasizing the role for PAR₂ in *A. alternata* filtrate activation.

DISCUSSION

Asthma is a chronic respiratory disease with significant cost, morbidity, and mortality. A critical factor in the development of allergic asthma is environmental allergen exposure and sensitization (3, 28, 35). Characteristics of allergic asthma include airway inflammation, mucin production, and airway remodeling, all of which may be affected by the interaction between the epithelium and environmental allergens. In this manuscript, we show that filtrates from an asthma-associated allergen (asthmagen), *A. alternata*, causes airway inflammation in mice, with lung cell recruitment that is dependent on filtrate protease activity. Using a human airway epithelial cell model, we show that serine-specific proteases from *A. alternata* filtrate directly activate human bronchial epithelial cells via the PAR₂. Furthermore, filtrate-mediated Ca²⁺ signaling in epithelial cells requires an active receptor and is dependent on the amount of PAR₂ expressed. Although this is the first report to demonstrate serine-proteases from *A. alternata* are sufficient to induce airway inflammation in nonsensitized animals, it is in agreement with a recent report demonstrating a role for PAR₂ in German cockroach frass-induced inflammation (32) and with several reports of an asthmagen-PAR₂ axis in inflammation in sensitized mouse models (9, 10, 36). These results highlight airway epithelial PAR₂ as a site of action for the environmental astmagens in the development of human allergic asthma.

Allergens as a group have very diverse characteristics, yet only a subset of allergens strongly associate with the development of asthma. A critical element separating astmagens, including those from cockroach, house dust mite, *A. alternata*, and *Penicillium citrinum*, from more benign allergens, is their association with robust protease activity. The proteolytic nature of these allergic pathogens can have direct effects on cells and tissues in the airway. Proteolytic targets include disruption of epithelial cell junctions, depletion of elastase inhibitors, cell receptor cleavage, alteration of salt and water movement, and inactivation of surfactant proteins (15, 38). These proteases can also have indirect effects, including providing foreign particulates access to underlying cells and tissues in the airway, or activating cellular signaling pathways via PARs (15, 22, 34, 35). Because activation of PARs can result in inflammatory, growth factor, or other signaling molecule release, they can contribute to some of the downstream physiological changes associated with allergic asthma, including immune cell recruitment, airway remodeling, or bronchoconstriction/bronchodilation. As such, protease interaction with the airway epithelium provides a first recognition of and response to allergen exposure and, thus, a potential site for astmagens to alter the local physiology and contribute to asthma.

A primary finding of our study is that *A. alternata* sensitization of the airway is protease-dependent and can directly induce inflammatory cell recruitment to the lung in the absence of sensitizing agents such as alum or ovalbumin. Similar findings have recently been reported from German cockroach-derived astmagens that initiated PAR₂ protease-dependent inflammation independent of sensitization (32). Because application of these proteases are intranasal and delivered under

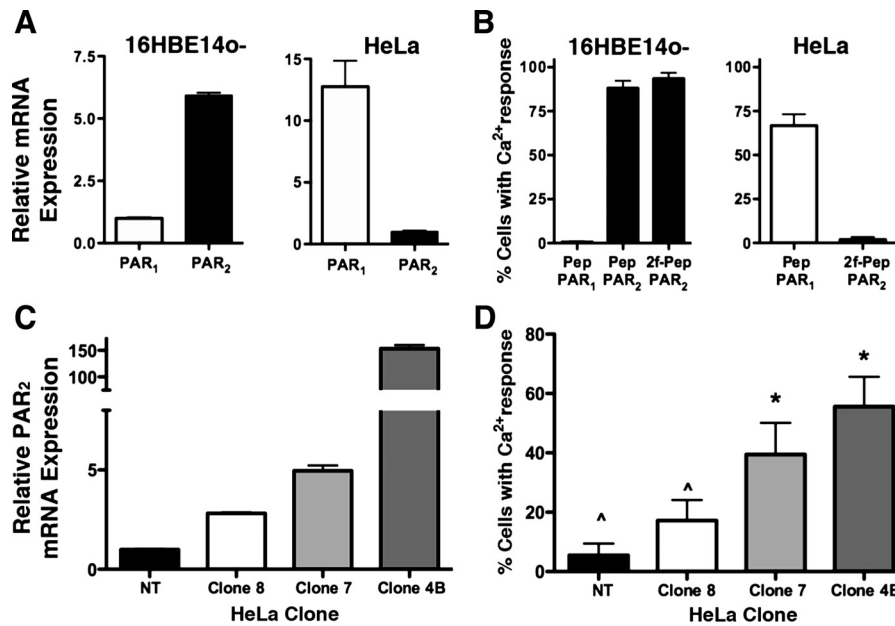


Fig. 8. *A. alternata*-induced $[Ca^{2+}]_i$ changes in epithelial cells are associated with PAR₂ expression. Comparative quantitation of PAR₂ mRNA expression was determined by real-time RT-PCR. **A:** relative expressions of PAR₁ and PAR₂ are shown for 16HBE14o- and nontransfected HeLa cells. 16HBE14o- data are presented as fold change in PAR₂/GAPDH expression (PAR₁/GAPDH expression set to 1). HeLa cell data are presented as fold change in PAR₁/GAPDH (PAR₂/GAPDH expression set to 1; note change in scale). **B:** Ca²⁺ responses to direct activation of PAR₁ with TFLRL-NH₂ (Pep PAR₁) or PAR₂ with SLIGRL-NH₂ (Pep PAR₂) or 2-furoyl-LIGRLO-NH₂ (2f-pep PAR₂) are shown for both 16HBE14o- cells (*left*) and HeLa cells (*right*). Consistent with PAR expression data in A, 50 μ M Pep PAR₁ application resulted in a limited Ca²⁺ response in 16HBE14o- cells ($0.7 \pm 0.4\%$), whereas 80 μ M Pep PAR₂ or 2.5 μ M 2f-Pep both resulted in robust Ca²⁺ responses (88.16 ± 4.1 and $93.59 \pm 3.3\%$, respectively). In contrast, 25 μ M Pep PAR₁ induced Ca²⁺ responses in $66.8 \pm 6.4\%$ of the HeLa cells, whereas 5 μ M of 2f-Pep induced a minimal Ca²⁺ response ($2.0 \pm 1.4\%$). **C:** real-time RT-PCR of PAR₂ mRNA expression for various HeLa clones displayed as fold change in PAR₂/GAPDH expression calibrated to nontransfected HeLa cells (NT). Clones were divided into the following categories: low (2- to 3-fold increase), medium (5- to 10-fold increase), and high (>10-fold expression) expression. The low-expression clone 8 displayed only a 2.8-fold increase in mRNA expression. The medium-expression clone 7 displayed a 5-fold increase in PAR₂ mRNA expression. The high-expression clone 4B displayed a 154-fold increase in PAR₂ mRNA compared with nontransfected HeLa cells. **D:** Ca²⁺ responses to *A. alternata* filtrate for each clone were executed as described in Fig. 5. HeLa transfectant Ca²⁺ response is dependent on the amount of PAR₂ expression. $\wedge$, Ca²⁺ response is significantly different from that observed for clone 4B. *, Ca²⁺ response is significantly different from that observed for nontransfected HeLa cells. Experiments are graphed $\pm$ SE; $n = 3$ for real-time RT-PCR; $n \geq 6$ for Ca²⁺ activation.

anesthesia to allow aspiration into the airway, we hypothesized that interaction with airway epithelial cells was likely the site of action of asthmagin proteases. The release of cytokines, chemokines, and other signaling molecules is a potential physiological outcome of PAR₂ activation that could be crucial in our murine model of *A. alternata* exposure. Consistent with this hypothesis, inflammatory cytokine release (TNF- α , IL-5, and IL-13) has been measured in animal models following tracheal instillation of PAR₂ activating peptide SLIGRL-NH₂ in ovalbumin-sensitized mice (9, 10).

Because the epithelium provides the first tissue of contact between asthmagin protease activity and the host cell, we more closely evaluated a human bronchial cell model (16HBE14o-cells) for protease-dependent *A. alternata* effects. Activation of PARs by exogenous or endogenous proteases occurs through the cleavage of the NH₂-terminus of the GPCR, which exposes a peptide sequence that can act as a receptor ligand (30, 34, 37). These tethered ligands then initiate several downstream signaling pathways. In the case of PAR₂, the primary signaling pathways include Ca²⁺, which occurs rapidly after activation, and mitogen-activated protein kinases (MAPK), which can occur over a more prolonged time course. Our report focuses on filtrates from *A. alternata* exposed to a human bronchial cell line that retains functional tight junctions in culture (39) and thus represents an airway cell model that best allows for apical

exposure of the filtrate, as would occur in vivo. It should be noted that previous reports on PAR expression in 16HBE14o-cells have provided evidence for all four PARs being expressed (1, 8). However, under the growth conditions presented in this manuscript, there is minimal expression of PAR₁, PAR₃, and PAR₄ and a corresponding minimal response to thrombin (Fig. 6), a known activator of all PARs except PAR₂. Thus 16HBE14o- cells provide an excellent airway cell model for examination of PAR₂ activation. We detail PAR₂ as a target for *A. alternata* proteases by monitoring rapid changes in $[Ca^{2+}]_i$. *A. alternata* also causes increases in MAPK, as measured by increased phosphorylated ERK 1/2 (data not shown). A variety of endogenous proteases have been associated with PAR₂ activation as well as defined and undefined allergen-associated proteases (35, 37, 38). Our results are consistent with serine protease(s) derived from *A. alternata* contributing to PAR₂ activation in human airway epithelial cells.

Airway epithelial cell models have been used in several previous reports to demonstrate inflammatory mediator release following PAR₂ activation by asthmagens, including *A. alternata*. Protease-dependent activation of an alveolar-derived human lung cell line, A549, by extracts from four fungi, including *A. alternata*, caused release of the proinflammatory cytokines IL-6 and IL-8 (19). Similar studies with A549 cells activated by Pen c 13, a protease derived from *P. citrinum*, (4)

or with the PAR₂-activating peptide SLIGKV-NH₂ (31) demonstrate IL-8 release. However, in both of these reports, it was also shown that PAR₁ could be important in IL-8 release. PAR₂ was involved in thymic stromal lymphopoietin (TSLP) release from a human bronchial epithelial cell line, BEAS-2B, following very high doses of *A. alternata* (50–100 µg/ml) extract (24). TSLP release was reduced by PAR₂ knockdown by transfection with small-interfering RNA targeted to PAR₂, consistent with a PAR₂-dependent release of TSLP. Although both serine and cysteine proteases could induce TSLP secretion from BEAS-2B cells, the *A. alternata* response was not blocked by a serine protease inhibitor, APMSF, but was partially blocked by a cysteine protease inhibitor, E-64. A similar response to high concentrations of *A. alternata* (50 µg/ml) generated PAR₂ responses in eosinophils that also were not blocked by serine protease inhibitor APMSF but were blocked by the aspartyl protease inhibitor pepstatin (25). In our experiments, extracts from *A. alternata* above 20 µg/ml lead to cytotoxicity of 16HBE14o- cells (data not shown), precluding accurate evaluations of these high concentrations of *A. alternata*. Furthermore, neither the cysteine protease inhibitor E-64 nor the aspartyl protease inhibitor pepstatin altered *A. alternata* filtrate-induced Ca²⁺ responses in 16HBE14o- cells used in the present study, suggesting that it is the serine protease activity from *A. alternata* that leads to airway epithelial activation. The differences observed in previous reports could be due to concentration and/or cytotoxicity effects. However, we cannot rule out differences in response to *A. alternata* due to cell type, which could include altered PAR₂ allele expression, accessory activating molecules, or G protein signaling, all shown to be important in defining PAR₂ signaling from different cell types (37). Further work will be needed to better clarify the differences between these model systems and how they impact in vivo response.

In this report, we show that mice exposed to the same *A. alternata* filtrates that activate PAR₂ in vitro result in protease-dependent inflammatory cell recruitment to the airways. These findings support our hypothesis that airway epithelial PAR₂ responses are involved in host responses to exogenous asthmagens, potentially leading to innate immune activation and inflammation that create symptoms associated with allergic asthma. However, activation of PAR₂ in a variety of animal and activation models has resulted in mixed results as to inflammation in asthma. For example, airway PAR₂ activation can result in proinflammatory effects such as we detail here (5, 9, 10, 32, 36), but also can result in anti-inflammatory and bronchorelaxant effects (6, 27). Similar, seemingly contradictory, findings can be found in cellular and tissue models (15). A full understanding of specific effects of PAR₂ activation will likely include the understanding of signaling pathways triggered under different conditions, coreceptor activation, cell type of activation, presence of antiproteases, as well as genetic backgrounds.

ACKNOWLEDGMENTS

We thank Dr. Rowena Campbell for background work on this project. We also thank Dr. Marilyn Halonen for constant conversations in moving these experiments forward, Dr. Barry Pryor for providing *A. alternata* filtrate, Daniel X. Sherwood for the alignpeaks program that allowed for Ca²⁺ data analysis, Dr. Heddwen Brooks for help with real-time RT-PCR, Dr. Josef Vagner for production of the 2-furoyl-LIGRLO-NH₂ compound, and Terri Boitano for help with manuscript preparation.

GRANTS

This work was funded in part through Semiconductor Research Corporation Project No. 425.024 and National Institutes of Health (NIH) Center Grant ES-06694 (S. Boitano); NIH Training Grant T32-HL-007249 (A. N. Flynn, C. L. Sherwood); Arizona Respiratory Center Startup Funds and NIH Grant AI-083403 (M. O. Daines). S. M. Schulz was a University of Arizona Undergraduate Biology Research Program scholar (HHMI 52005889).

DISCLOSURES

No conflicts of interest are declared by the authors.

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