



Per a 10 activates human derived epithelial cell line in a protease dependent manner via PAR-2

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ABSTRACT

Background: Protease activity of Per a 10 has been shown to modulate dendritic cells toward Th-2 polarization and to induce airway inflammation.

Objective: To elucidate the role of serine protease activity of Per a 10 in inducing biochemical responses in epithelial cells.

Methods: Per a 10 was inactivated by heat treatment (Δ Per a 10) or AEBSF (iPer a 10). A549 cells were exposed to either enzymatically active/inactive Per a 10. The supernatant was analyzed for the secretion of proinflammatory cytokines by ELISA. Ca²⁺ mobilization was analyzed by flow cytometry. A PAR-2 derived synthetic peptide 28GTNRSSKGRSLIGKVDGTSHTGKGVTC54 was incubated with Per a 10 and the resultant cleaved products were analyzed by LC-MS. PAR-2 activation was inhibited by PAR-2 cleavage inhibiting antibody.

Results: Δ Per a 10 was completely inactivated whereas iPer a 10 showed some residual activity. nPer a 10 having protease activity increased the secretion of IL-6, IL-8 and GM-CSF from A549 in a dose and time dependent manner whereas iPer a 10 has reduced cytokine secretion. Δ Per a 10 and rPer a 10 were unable to activate the cells. nPer a 10 mobilized intracellular Ca²⁺. nPer a 10 cleaved the PAR-2 derived peptide between arginine and serine residues (36R–S37) to expose PAR-2 ligand SLIGKV, as determined by LC-MS. Incubating with anti-PAR-2 cleavage antibody showed diminished cytokine secretion when treated with nPer a 10.

Conclusion: Serine protease activity of Per a 10 activates A549 cells to secrete proinflammatory cytokines by PAR-2 activation and Ca²⁺ mobilization and can be exploited therapeutically.

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Introduction

Exposure to cockroach allergens at home is a major risk factor associated with allergy and asthma in sensitized individuals (Arruda and Chapman 2001; Arizmendi et al. 2011). *Blattella germanica* and *Periplaneta americana* are the two principle domiciliary species of cockroaches (Sookrung and Chaicumpa 2010). *P. americana* contains 22 IgE binding components of which a few major allergens namely Per a 1, Per a 3 (Arylphorins), Per a 6 (troponin-C), Per a 7 (tropomyosin), Per a 9 (arginine kinase) and Per a 10

(serine protease) have been isolated and characterized by various methods (Sudha et al. 2008; Pomes et al. 2007).

Proteases play an important role in eliciting IgE responses and consequent development of allergic reactions (Saw et al. 2012; Kheradmand et al. 2002; Tripathi et al. 2009; Kukreja et al. 2008). Cockroach proteases contribute to allergic inflammation and have been shown to increase bronchial airway epithelial permeability (Antony et al. 2002). Serine protease activity of Per a 10 induces Th-2 responses and plays an important role in augmenting allergic airway inflammation in mice. Further it acts as an adjuvant for bystander allergen by virtue of its proteolytic activity (Sudha et al. 2009). Proteolytic activity of Per a 10 modulates dendritic cell polarization of T-cells toward Th-2 by CD86 upregulation (Goel et al. 2012).

Airway epithelial cells are the first line of defense and act as physical/chemical barrier to shield the immune cells from the inhaled antigens. They express various pattern recognition receptors to sense the external environment and initiate and orchestrate local inflammatory responses by secreting proinflammatory

Abbreviations: AEBSF-4, benzenesulfonyl fluoride hydrochloride; BSA, bovine serum albumin; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage colony-stimulating factor; PAR, protease activated receptor.

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cytokines. Protease activated receptors (PARs) are G protein coupled receptors encompassing a family of four receptors PAR-1 to PAR-4 (Hollenberg 1999). PARs have a unique mechanism of activation, where proteinases cleave within an N-terminus domain to unmask a tethered ligand which then binds and activates the receptor. PAR-2 senses the environment for serine proteinases and has been implicated in development of allergic inflammatory reactions (Arizmendi et al. 2011). PAR-2 is expressed by a variety of cells including both structural and immune cells and several reports have linked PAR-2 activation to allergic sensitization (Stenton et al. 2002; Schmidlin et al. 2001; Hamilton et al. 2001). The asthmatic respiratory epithelium has been shown to secrete chemokines and proinflammatory cytokines relevant to asthma pathophysiology (Barnes 2008). But the exact mechanism of this release is not understood precisely. In the present study we examined the role of serine protease activity of Per a 10 on airway epithelial cell activation. We compared the epithelial cell response, incubated with active native Per a 10 and a partially inactive AEBSF treated Per a 10 to that of inactive heat denatured and recombinant Per a 10, in terms of cytokine release and cell surface receptor activation.

Materials and methods

Reagents

Azocollagen, RPMI-1640, penicillin–streptomycin (Sigma, St. Louis, MO, USA), Zymefree cell dissociation solution (Himedia, Mumbai, India), FBS (Gibco, Carlsbad, CA, USA), AEBSF, Fluoro-4AM (Invitrogen, Carlsbad, CA, USA), PAR-2 peptide (GL Biochem, Shanghai, China), SAM11; anti-PAR2 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), ELISA kits GMCSF (BD pharmlingen, San Diego, CA, USA), IL-6 and IL-8 (eBiosciences, San Diego, CA, USA).

Isolation of native and recombinant Per a 10

Per a 10 was isolated from whole body cockroach extract prepared in PBS by affinity chromatography (Sudha et al. 2008). Recombinant Per a 10 (rPer a 10) was purified as described elsewhere (Govindaraj et al. 2013).

Proteolytic activity determination

Enzymatic activity of Per a 10 was determined qualitatively by gelatin zymography and quantitatively by azocollagen assay as previously described (Goel et al. 2012; Govindaraj et al. 2013). Briefly, protein was fractionated on 12.5% native PAGE containing 1% gelatin in resolving gel, washed with 2.5% triton $\times$ 100 for 10 min and rinsed twice with deionised distilled water. The gel was incubated overnight in 100 mM glycine (pH 7.4) and stained with commassie brilliant blue. Clear bands represented protease activity. For azocollagen assay 100 μ l (10 mg/ml) of azocollagen in Tris–HCl buffer (pH 7.4) was incubated with 2 μ g of Per a 10 at 37 °C for 60 min. Reaction was stopped by adding 4% (v/v) trichloroacetic acid, centrifuged and absorbance of supernatant was measured at 520 nm. Azocollagen without Per a 10 served as negative control.

Inhibition experiments

To inhibit protease activity of Per a 10, it was either heated at 90 °C for 90 min or treated with serine protease inhibitor AEBSF (10 mM for 60 min). Protease activated receptors were inhibited by incubating A549 cells with either 4 μ g/ml SAM11 (a PAR-2 inhibiting monoclonal antibody) or ATAP2 (PAR-1 inhibiting monoclonal antibody) for 2 h before the stimulation.

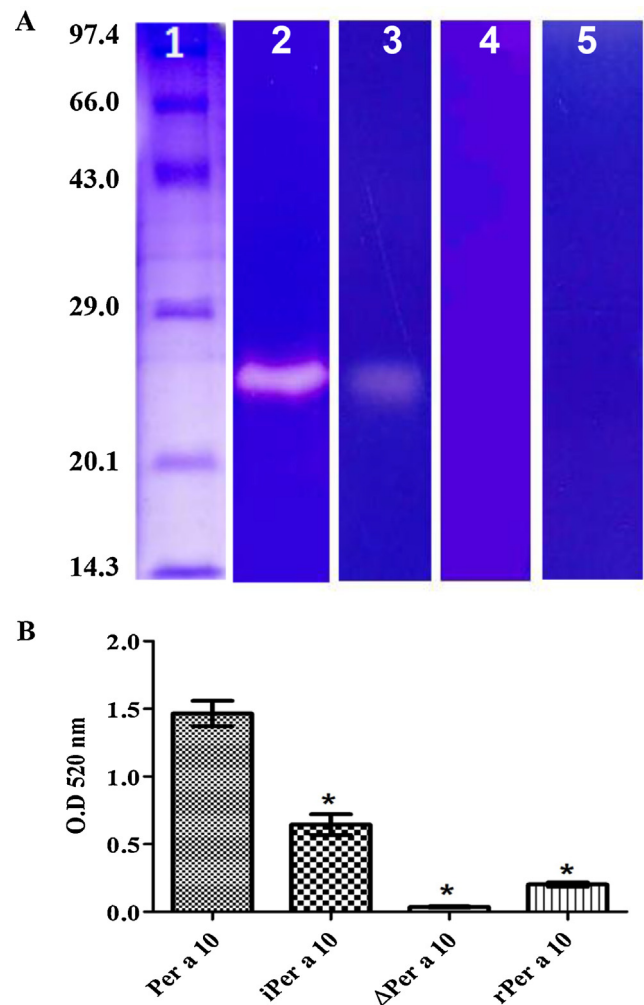


Fig. 1. Proteolytic activity assays. For gelatin zymography 2 μ g each of Per a 10 (lane 2), iPer a 10 (lane 3), rPer a 10 and Δ Per a 10 (lanes 4 and 5 respectively) were resolved by Native -PAGE with 1% gelatin in the resolving gel. (b) proteolytic activity of Per a 10, iPer a 10, Δ Per a 10 and rPer a 10 was also assessed quantitatively with azocollagen as a substrate. * P < 0.05 compared with Per a 10.

Cell culture and antigen exposure

A549 cells were cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37 °C in 5% CO₂. Cells were passaged in zymefree cell dissociation solution to minimize proteolytic activation of PARs, seeded in 24 well tissue culture plates and grown till 80% confluency. Cells were incubated in serum free media for 24 h and then stimulated with different concentrations of nPer a 10. To check the effect of Per a 10 activity, A549 cells were exposed either to 10 μ g of iPer a 10 (AEBSF inactivated), rPer a 10 (recombinant) or Δ Per a 10 (heat inactivated) for 24 h. Culture supernatant was collected, centrifuged at 1200 $\times$ g for 5 min and stored at –20 °C for further analysis.

Determination of cytokines by enzyme linked immunosorbent assay (ELISA)

The levels of proinflammatory cytokines IL-6, IL-8 and GMCSF in the culture supernatant were determined using paired antibodies as per the manufacturer's instruction (eBiosciences for IL-6 and IL-8 and BD Biosciences for GMCSF).

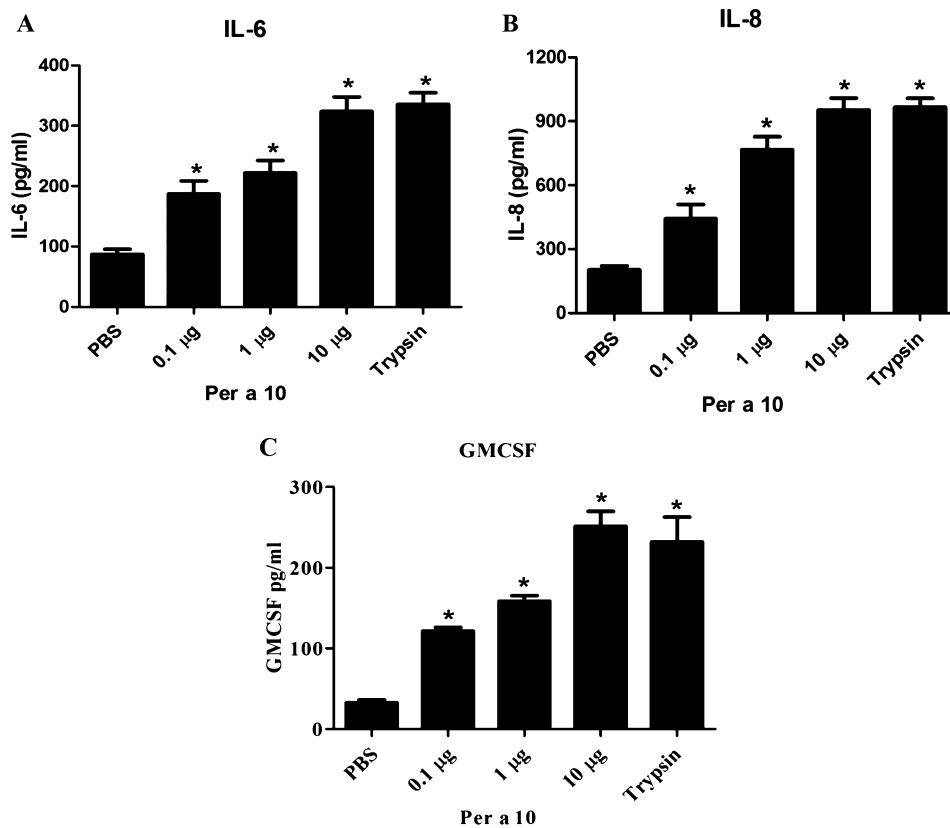


Fig. 2. nPer a 10 modulates secretion of proinflammatory cytokines (a) IL-6, (b) IL-8 and (c) GMCSF by airway epithelial cells in a dose dependent manner. Cytokine levels were evaluated 24 h after stimulation, in supernatants from A549 cells stimulated with PBS, trypsin (1 ng) or nPer a 10 (0.01–10 µg). * $P < 0.05$ compared with PBS stimulated cells.

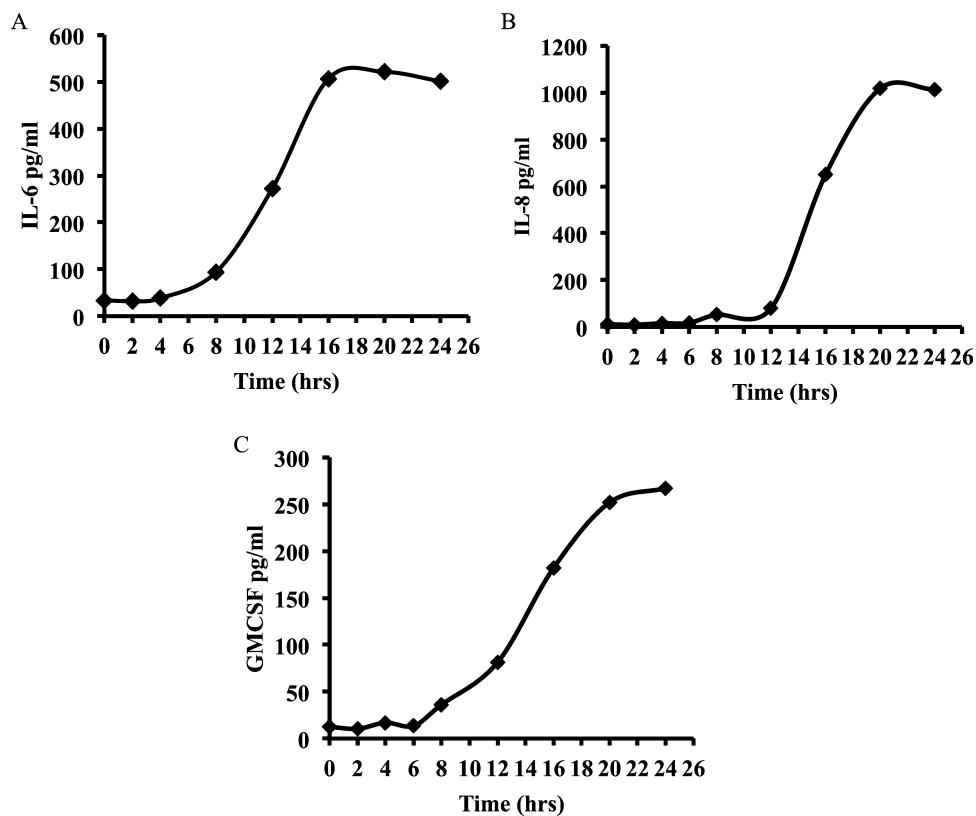


Fig. 3. Time dependent secretion of (a) IL-6, (b) IL-8 and (c) GMCSF when incubated with 10 µg of nPer a 10.

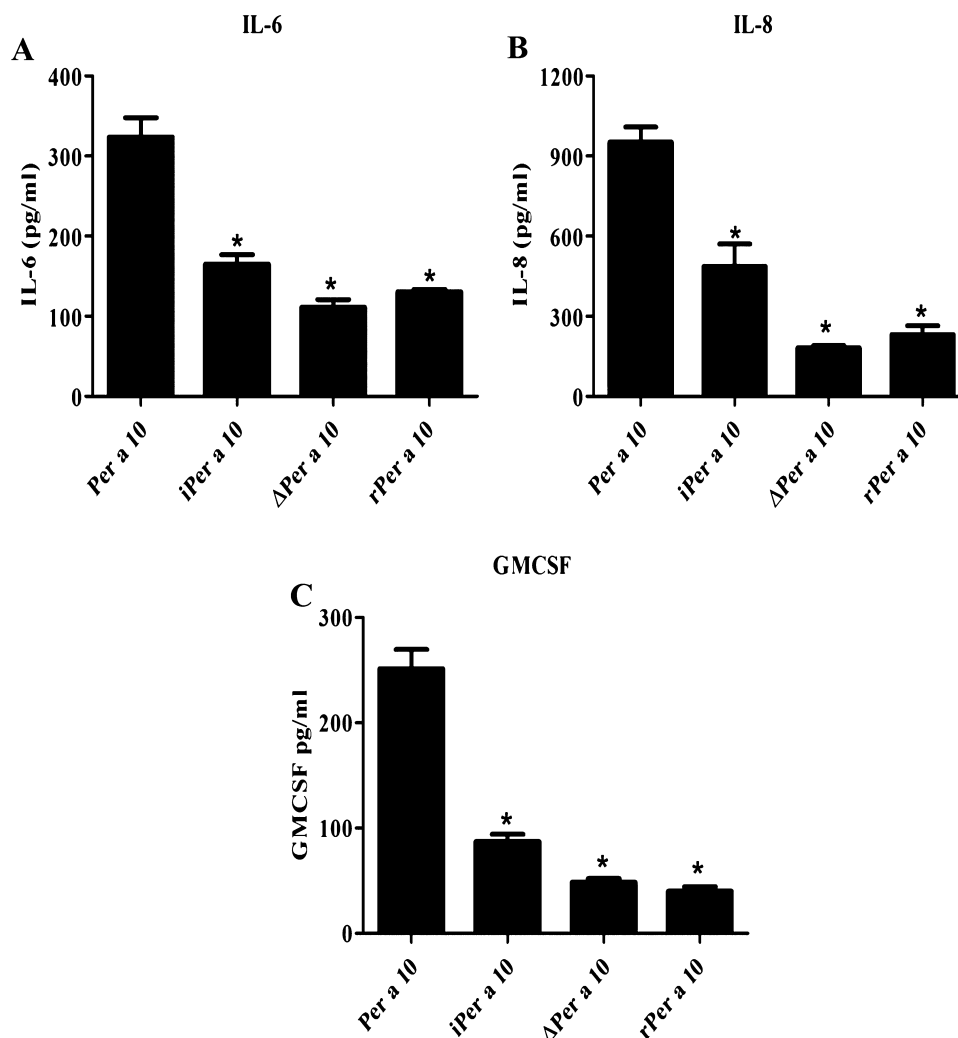


Fig. 4. The effect of proteolytic activity of Per a 10 on cytokine release by A549 cells. A549 cells were grown till confluency and serum starved for 24 hrs. The serum starved cells were then exposed to 10 μ g of either nPer a 10, iPer a 10, Δ Per a 10 or rPer a 10. The supernatant was then assessed for secretion of (a) IL-6, (b) IL-8 and (c) GM-CSF. * $P < 0.05$ compared with Per a 10 stimulated cells.

Cleavage of PAR-2 receptor

A 27mer peptide “GTNRSSKGRSLIGKVDGTSHVTGKGVT” representing the cleavage/activation site of human PAR-2 receptor was synthesized. The 27 mer peptide was incubated with Per a 10 for 45 min at 37 °C and then subjected to LC-MS. Peptide fragments resulting from Per a 10 hydrolysis were determined by analysis of the respective masses of the fragments isolated by HPLC.

Flow cytometry

A549 cells grown till 80% confluency were detached using cell dissociation medium blocked for 1 h at 4 °C with PBS containing 0.1% (w/v) BSA. Cells were then stained either with mouse anti human PAR-1 or mouse anti human PAR-2 antibody for 2 h and Alexa flour 488 labeled goat anti-mouse IgG for half an hour. Cells were then washed with FACS buffer (PBS + 0.1% (w/v) BSA) and PAR expression was analyzed by flow cytometer.

Intracellular Ca^{2+} mobilization

Effect of Per a 10 on intracellular Ca^{2+} levels of A549 cells was assessed using a fluorescent Ca^{2+} binding dye Fluo-4AM. Briefly

A549 cells were grown till 80% confluency and harvested using zymefree cell dissociation solution, pelleted by centrifugation and resuspended in serum free RPMI-1640 and then loaded with Fluo-4 AM for 45 min at 37 °C. The cells (1×10^6 per ml) were exposed either to nPer a 10 or PBS alone. The change in fluorescence at excitation/emission of 494/506 nm was analyzed by flow cytometer.

Data analysis

All the experiments were carried out at least three times unless otherwise stated. Data are expressed as mean $\pm$ SEM. Statistical significance between means was determined using one way ANOVA in GraphPad Prism software. p values of <0.05 were considered significant.

Results

Per a 10 induces secretion of proinflammatory cytokines from A549 cells

Per a 10 was purified to homogeneity from cockroach extract by affinity chromatography and resolved as a single band of 28 kDa on SDS-PAGE. Its activity was assessed qualitatively by gelatin

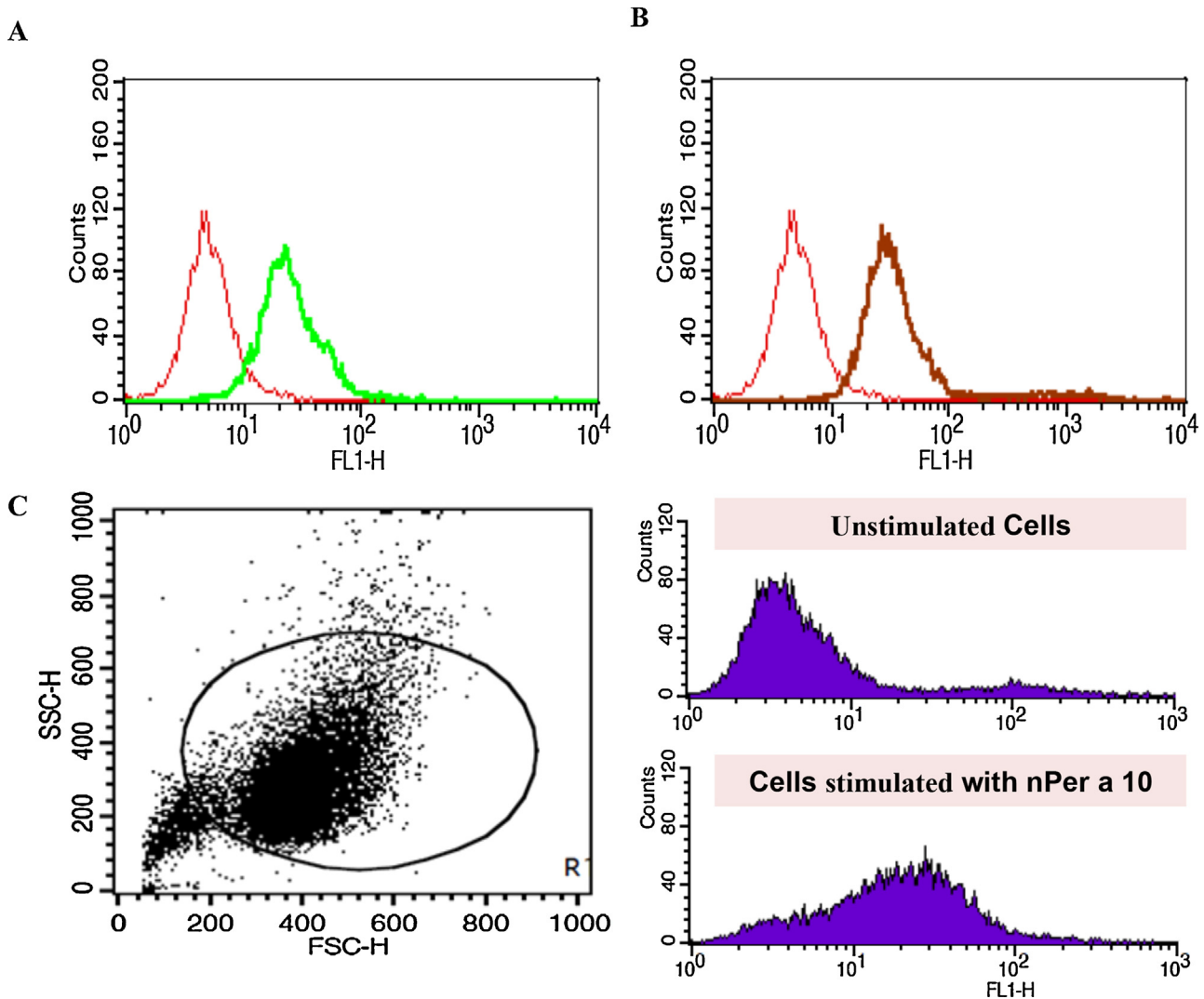


Fig. 5. Flow cytometric analysis of PAR expression and nPer a 10 induced intracellular Ca^{2+} mobilization in A549 cells. A549 cells were cultured in RPMI-1640 supplemented with 10% FCS and incubated with anti human PAR-1 or PAR-2 antibody. The cells were then stained with Alexa flour 488 labeled anti mouse secondary antibody and analyzed by flow. (a) PAR-1 expression (b) PAR-2 expression. A549 cells were incubated with Fluo-4AM for 30 min and then stimulated with nPer a 10, the change in fluorescence due to Ca^{2+} mobilization was measured using flow cytometry at excitation and emission of 494/506 nm respectively. (c) SSC versus FSC of A549 cells where R shows the gated population and histogram of untreated and cells treated with nPer a 10.

zymography as a clear band as shown in Fig. 1A and quantitatively by azocollagen assay (Fig. 1B). To determine whether Per a 10 induces secretion of proinflammatory cytokines from human airway derived epithelial cell line A549, serum starved A549 cells were exposed to native active Per a 10 (nPer a 10) at various concentrations and levels of proinflammatory cytokines secreted in the supernatant were measured by ELISA. As shown in Fig. 2 dose dependent increase in secretion of IL-6, IL-8 and GM-CSF was observed after 24 h of exposure. Significant cytokine levels above background levels were observed at Per a 10 concentrations as low as 100 ng. Time dependent secretion of these cytokines was determined by 10 μg of nPer a 10 on A549 cell cultures (Fig. 3).

Per a 10 activates A549 cells in an activity dependent manner

Recombinant Per a 10 (rPer a 10) was purified and resolved as a single band of 28 kDa on 12.5% SDS-PAGE. Heat inactivated Per a 10 (Δ Per a 10) and rPer a 10 did not show any band on gelatin zymography gel, whereas AEBSF inactivated Per a 10 showed residual activity as a faint colorless band (Fig. 1A). The activity of rPer a 10, Δ Per a 10 and iPer a 10 was confirmed quantitatively by azocollagen assay as shown in Fig. 1B.

To ascertain that this stimulatory effect of Per a 10 was due to its serine protease activity, A549 cells were exposed to AEBSF treated, heat treated and recombinant Per a 10 for 24 h and the supernatant was analyzed for secretion of the above said proinflammatory cytokines. Heat treatment of Per a 10 completely abolished its stimulatory effects. Heat treatment abolishes the tertiary structure of a protein and affects its biochemical functioning. Δ Per a 10 retains its primary structure but lost its protease activity and was unable to induce its effects on the airway epithelial cells as shown in Fig. 3. Improper folding or conformational changes may have rendered rPer a 10 inactive and also failed to activate A549 cells. There was a significant lowered secretion of IL-6, IL-8 and GM-CSF in supernatant of iPer a 10 exposed cells, owing to its residual protease activity. AEBSF failed to completely inactivate Per a 10 which resulted in diminished but significant secretion of IL-6, IL-8 and GM-CSF by iPer a 10 treated cells (Fig. 4).

PAR-2 activation is sufficient for Per a 10 mediated activation of A549 cells

We checked for expression of PAR-1 and PAR-2 on A549 cells. As shown in Fig. 5A and B. A549 cells do express PAR-1 and PAR-2

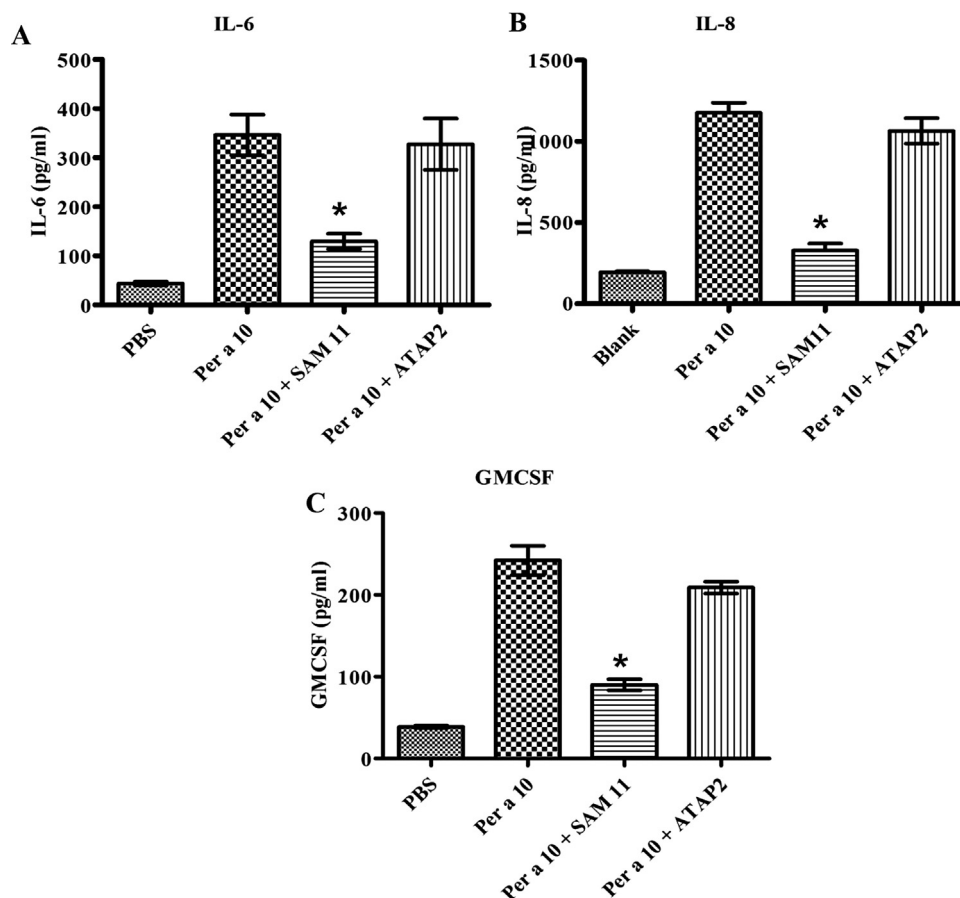


Fig. 6. Effect of anti-PAR antibodies on Per a 10 induced cytokine secretion by A549 cells. A549 cells were preincubated with 4 μ g of either PAR-2 inhibiting antibody (SAM11) or PAR-1 inhibiting antibody (ATAP-2) at 37 °C for 2 hr and then exposed to nPer a 10 for 24 hr, after which (a) IL-6 and (b) IL-8 and (c) GMCSF was measured in the culture supernatant. * $P < 0.05$ compared with Per a 10 stimulated cells.

receptors. We next investigated whether PAR-2 inhibition has an effect on Per a 10 induced cytokine secretion from A549 cells. We treated the serum starved A549 cells either with PAR-1 (ATAP2) or PAR-2 (SAM11) receptor cleavage blocking antibody. As shown in Fig. 6, 4 μ g/ml of SAM11 significantly reduced the Per a 10 induced secretion of IL-6, IL-8 and GMCSF. Preincubation of cells with PAR-1 blocking antibody had no significant effect on cytokine release. This showed that Per a 10 causes the activation of A549 cells and release of proinflammatory cytokines via PAR-2 receptors.

Native active Per a 10 cleaves PAR-2 receptor derived peptide

To determine whether Per a 10 cleaves PAR-2 receptor, peptide containing the cleavage/activation site of PAR-2 receptor (GTNRSSKGRSLIGKVDGTSHTGKGVT) was synthesized and incubated with native active Per a 10 at 37 °C for 45 min. The resulting proteolytic fragments were isolated and characterized using liquid chromatography and mass spectrometry.

nPer a 10 cleaved the PAR-2 derived peptide between serine and arginine residues (Arg³⁶-Ser³⁷) as shown in Fig. 7. This exposed the tethered receptor ligand domain SLIGKV that is sufficient for the activation of PAR-2. This demonstrated that Per a 10 is capable to unmask the tethered ligand SLIGKV of PAR-2 receptor and has a potential to activate it.

Native active Per a 10 mobilizes intracellular calcium

Our results show that serine protease activity of Per a 10 have a role in activating airway epithelial cells and that this activation

might be through PAR-2 activation. Cleavage and activation of PAR-2 receptors has been shown to mobilize intracellular Ca^{2+} . So in order to determine the effect of nPer a 10 on intracellular Ca^{2+} levels, A549 cells loaded with fluo-4AM, a Ca^{2+} sensitive fluorescent dye exposed either to nPer a 10 or PBS were analyzed for an increased fluorescence using flow cytometer. The mean fluorescence intensity of Per a 10 exposed cells was more than the PBS exposed cells as shown in Fig. 6.

Discussion

There is mounting evidence suggesting that allergens possessing intrinsic biochemical activity can play an important role in allergic sensitization and eliciting inflammatory responses at bronchial surfaces by activating airway epithelial cells. Certain potent allergens from house dust mite, fungi, pollens and cockroaches have been shown to possess intrinsic proteolytic activity by the virtue of which they can directly interact with host immune structures. Although studies have been carried out on effect of proteases in cockroach extract on immunological structures none of them included individual proteases and its effects in isolation (Page et al. 2005; Hong et al. 2004). The present study focused on elucidating the role of serine protease activity of Per a 10 on airway epithelial activation and to gain an insight into its possible mechanism.

The levels of inflammatory cytokines like IL-6, IL-8 and GMCSF are increased in sputum of asthmatic patients and are known to play a significant role in pathophysiology of allergic diseases (Hoshi et al. 1995; Mukaida 2003). Further Airway epithelial cells have been implicated in allergic reactions and are known to orchestrate

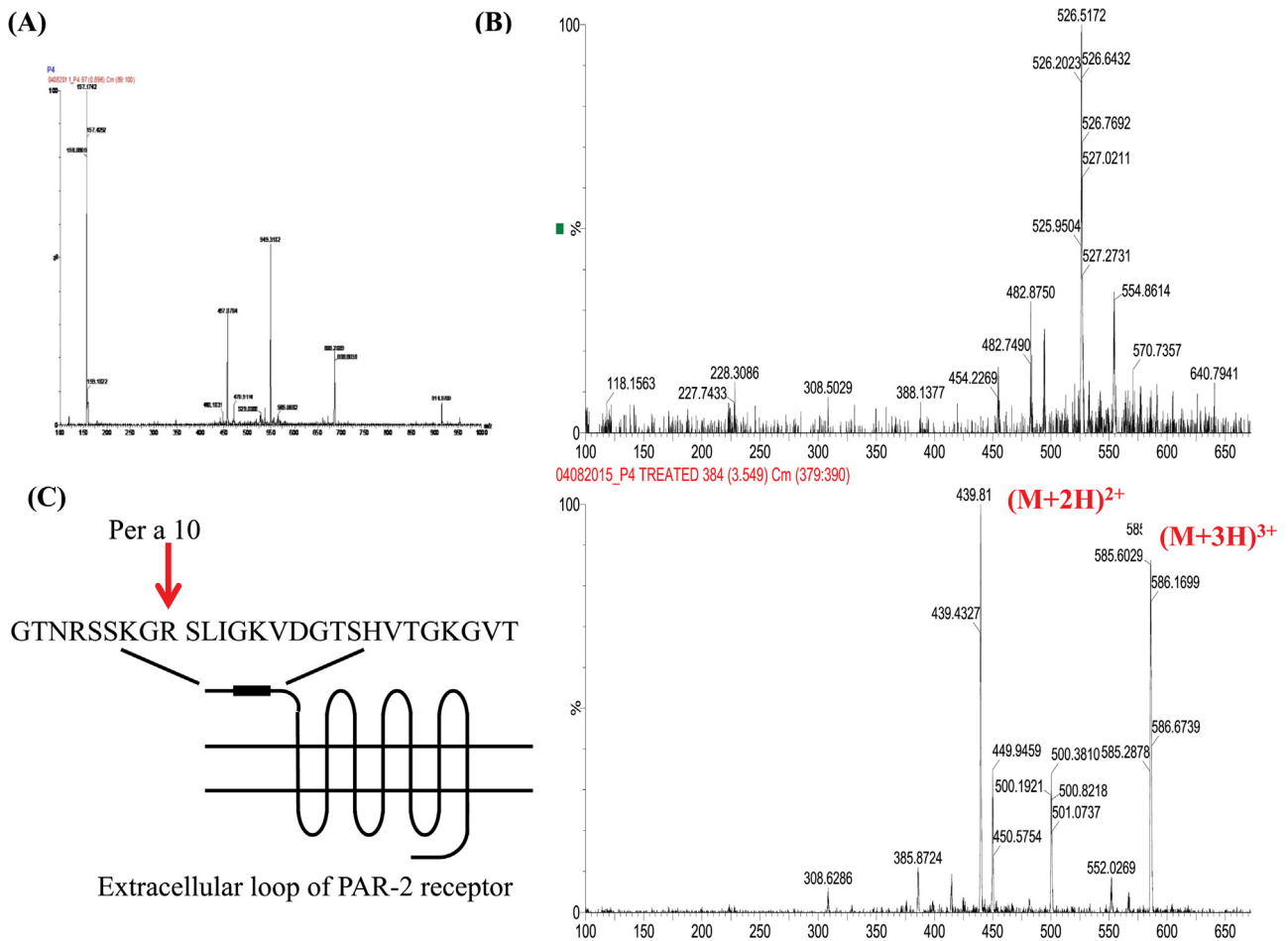


Fig. 7. Per a 10 cleavage site on PAR-2 derived cleavage/activation sequence. A peptide containing the activation/cleavage sequence of human PAR-2 receptor was incubated with Per a 10 and subsequent cleavage products were analyzed by LCMS. (a) MS of uncleaved peptide, (b) MS of peptide treated with Per a 10 and (c) potential cleavage site.

local inflammatory responses (Kale and Arora 2013). Previously airway epithelial cells have been used in many studies to examine mediator release following exposure to proteases. To determine if Per a 10 is able to activate A549 cells we stimulated serum starved A549 cells with native active Per a 10 (nPer a 10), and analyzed the supernatant for secretion of proinflammatory cytokines IL-6, IL-8 and GMCSF. IL-8 is a potent eosinophil and neutrophil chemoattractant, GMCSF enhances antigen presentation by dendritic cells and acts as an eosinophil chemoattractant whereas IL-6 plays an important role in promoting differentiation of CD4⁺ T cells to Th-2 cells (Neveu et al. 2010). Stimulation of alveolar A549 cells with native active Per a 10 (nPer a 10) triggered the release of proinflammatory cytokines IL-6, IL-8 and GMCSF in a dose and time dependent manner. Further there was a significant increase in release of IL-6 and IL-8 after 8 h of stimulation whereas GMCSF secretion increased significantly only after 12 h of incubation. Our results are consistent with previous studies where exposure of A549 cells to house dust mite proteases stimulates the secretion of proinflammatory cytokines (King et al. 1998). Also Sun et al., demonstrated release of GMCSF and eotaxin from human pulmonary epithelial cells exposed to Der p 3 and Der p 9 (Sun et al. 2001). Similar studies with *A. alternata* extract and Pen c 13 exposed A549 cells determined proinflammatory cytokine release.

To further ascertain whether serine protease activity of Per a 10 is required for activation of airway epithelial cells we used three variants, iPer 10, ΔPer a 10 and rPer a 10 of native active Per a 10. iPer a 10 was treated with serine protease inhibitor AEBF for 1 h and retained tertiary structure of nPer a 10 and some residual

protease activity. ΔPer a 10 was heat inactivated at 90 °C for 90 min and lacked any protease activity as heat treatment disrupts tertiary structure, whereas rPer a 10 was inactive and probably misfolded (Govindaraj et al. 2013). A549 cells exposed to iPer a 10 showed decreased but significant release of proinflammatory cytokines. This can be due to its residual protease activity as evident from zymography and azo-collagen assay (Fig. 1). Proteolytically inactive ΔPer a 10 and rPer a 10 failed to activate A549 cells. Together these results suggest that protease activity of Per a 10 is responsible for the release of proinflammatory cytokines from airway epithelial cell line A549, and may play a prominent role in initiating inflammatory responses at mucosal surfaces in allergic patients. There is an increasing amount of evidence depicting that apart from genetic predisposition intrinsic biochemical properties of allergens may contribute and promote allergic sensitizations at mucosal surfaces.

The next objective of this study was to ascertain the mechanism of release of proinflammatory cytokines from the airway epithelial cells. Airway epithelial cells express various receptors (PARs, TLRs, NOD like receptors) that sense the inhaled environment for danger (Lambrecht and Hammad 2012). Of these, PAR-2 and TLR-4 have been implicated in allergic diseases. PAR-2 belongs to a unique GPCR family and have specific cleavage sites recognized by various proteases on their extracellular N-terminal domain. Proteases initiate signaling by cleavage within the extracellular N-terminal region to form a tethered ligand which then binds intramolecularly and activates the receptor (Brass and Molino 1997). Specific activation of PARs has been shown to regulate the production of IL-6, IL-8 and prostaglandin E2 in A549 and BEAS-2B cells

(Asokanathan et al. 2002). Proteases from house dust mite, fungi and pollens have shown to interact with PAR-2. As Per a 10 has a serine protease activity we hypothesized it may work through activating PAR-2 receptors. To predict the potential cleavage sites, sequences corresponding to the N-terminal sequences have been used (Adam et al. 2006). We synthesized a 27mer peptide corresponding to the N-terminal sequence of human PAR-2. nPer a 10 cleaves PAR-2 receptor at a specific site and unmasks a tethered ligand, SLIGKV indicating that Per a 10 has a potential to activate PAR-2. Activation of PAR receptors leads to cellular activation and biochemical changes via inositol phospholipid activation and Ca^{2+} mobilization. Intracellular calcium levels were high in nPer a 10 exposed A549 cells than the control which is in accordance with previous reports where interaction of proteases with PAR receptors has shown to trigger Ca^{2+} mobilization from internal stores to cytoplasm (Kawabata et al. 1999). We further reasoned that if Per a 10 activates airway epithelial cells via, activating PAR-2 receptors, blocking of these receptors should dampen the response. We first confirmed the expression of PAR-1 and PAR-2 on A549 cells using PAR specific antibodies and flow cytometry. Using PAR-1 and PAR-2 receptor cleavage blocking antibody the cleavage of PAR receptors was blocked. A549 cells in which PAR-2 receptor activation was blocked showed diminished release of proinflammatory cytokines from the epithelial cells; whereas blocking PAR-1 had no significant change in release of these cytokines. These results are in accordance with previous studies where PAR-2 blocking antibodies considerably lowered AHR, eosinophil infiltration (Fujisawa et al. 2008) and serum IgG1 antibodies.

In conclusion our data suggests that Per a 10 activates airway epithelial cells and induces secretion of proinflammatory cytokines IL-6, IL-8 and GM-CSF in an activity and time dependent manner. This activation of epithelial cells is mediated through cleavage of PAR-2 receptors on airway epithelial cells. Further studies are needed to investigate the better understanding of protease-PAR-2 interactions as it can be intervened therapeutically to subvert allergic manifestations. This finding indicates that serine protease activity of Per a 10 is a major contributor of its allergenicity and that proteolytically inactive rPer a 10 can be explored further for immunotherapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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