Innate Generation of Thrombin and Intracellular Oxidants in Airway Epithelium by Allergen Der p 1

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This work was supported by the Wellcome Trust (Award 087650, to CR).

Disclosure of potential conflict of interest: The authors declare that they have no conflicts of interest.
**Capsule Summary**

Innate responses to allergens are key to allergy development, but poorly understood. Unexpectedly, and convergent with TLR3 signaling, Der p 1 directly activates prothrombin and generates intracellular oxidants, suggesting novel innate mechanisms for disease progression.

**Keywords:** House dust mite allergen; reactive oxidant species; airway epithelial cells; allergen delivery inhibitor; innate immune receptors; pannexon channels; thrombin
To the Editor:

Group 1 cysteine protease allergens from house dust mites (HDMs) are targets of a new class of drugs known as Allergen Delivery Inhibitors (ADIs) which are entering development for asthma therapy.\(^1\) In studying pro-inflammatory signaling by protease allergens, attention has focused on their direct cleavage of protease-activated receptors (PARs).\(^2\) We have explored an alternative view, namely that a key HDM allergen triggers the activation of thrombin and stimulates production of intracellular reactive oxygen species (ROS) and the extracellular release of ATP. ROS have significance because they orchestrate an allergic polarization of immune responses, and both heightened ROS production and a broad deficit in anti-oxidant defenses are characteristics of asthma.\(^3\)

To investigate the production of intracellular ROS we loaded human airway epithelial cells (primary cultures and established lines) with dihydrorhodamine 123 and exposed them to a natural mixture of *D. pteronyssinus* allergens. This resulted in a sustained generation of ROS (Fig 1A,B) associated with mitochondria and nuclei (see Methods and Fig 1C,D and Fig E1a-d in this article’s online repository at www.jacionline.org).

ADZ 51,457 and ADZ 51,529, which are reversible ADIs targeting Group 1 HDM protease allergens\(^1\), substantially reduced ROS generation (Fig 1E). Purified natural Der p 1 replicated ROS production and was fully inhibited by ADZ 51,457 (Fig 1F). In contrast, an irreversible inhibitor of serine proteases had no effect on ROS production and purified Der p 2 conspicuously failed to elicit ROS generation (Fig E2a,b in this article’s online repository at www.jacionline.org). Thus, among natural HDM allergens the initiators of intracellular ROS generation are the Group 1 cysteine proteases.

Surprisingly, ROS production by HDM allergens was transduced through PAR1 and PAR4, with only a small contribution from PAR2 (Fig 1G,H, and Fig E3a-e in this article’s online repository at www.jacionline.org). These responses required the opening of pannexons which are *inter alia* conduits for ATP release (Fig 1I). Interestingly, the viral RNA surrogate polyinosinic:polycytidylic acid (poly i:c) also caused pannexon-dependent ROS production (Fig 1I). Although HDM allergens and poly i:c initiated ROS production differently (Fig E4a,b in this article’s online repository at www.jacionline.org), their signaling converges at pannexons (Fig. 1I) with the extracellular release of
ATP and activation of mechanisms sensitive to the allosteric P$_2$X$_7$ receptor modulator, AZ 10606120 (Fig 2A,B).

Stimulation of PAR1 and PAR4 has not previously been associated with Der p 1, so we were interested in determining whether this involved the generation of thrombin, their canonical activator. The thrombin inhibitor argatroban inhibited ROS generation by HDM allergens, whereas the Factor Xa inhibitor apixaban was without effect (Fig 2C,D and Fig E5a-c in this article’s online repository at www.jacionline.org), thus excluding thrombin formation by the full coagulation cascade. Interestingly, both argatroban and a PAR1 antagonist were effective inhibitors of poly i:c (Fig 2E,F).

Incubation of prothrombin with mixed HDM allergens caused the appearance of prethrombin-1, the zymogen form of meizothrombin desF1, and the B chain of thrombin as major products. This process was inhibited by ADZ 50,000, an irreversible active site titrant analogue of ADZ 51,457 and ADZ 51,529 (Fig 2G). Formation of thrombin by Der p 1 provides further insight into the PAR siRNA data (Fig 1G,H) and a possible explanation of the extensive antagonism of ROS formation by PAR1 antagonists (SCH 79797, FR 171113) and the PAR4 antagonist, tcY-NH$_2$ (see Fig E3a-c in this article’s online repository at www.jacionline.org). Heterodimerization of PAR1 and PAR4 is preceded, providing a mechanism for thrombin bound to PAR1 through exosite 1 to cleave PAR4 (which cannot bind) more efficiently. The formation of a ternary complex would thus render ROS generation sensitive to antagonism of both receptors and imply that the main effector of Der p 1-stimulated ROS production might be PAR4, which is notably associated with epithelial-mesenchymal transition in airway cells.

Hitherto, PAR1 and PAR4 have not been considered activatable by Group 1 HDM allergens, but in revealing the Der p 1-dependent cleavage of prothrombin we have identified their canonical activation with subsequent intracellular ROS formation via ATP release. Extracellular ATP is elevated in asthma, which is noteworthy because it stimulates dendritic cells and triggers the release of IL-33, which is genetically linked to asthma susceptibility and a key activator of cytokine production by iH$_2$ nuocytes. Thrombin is present in airway surface liquid in asthma at levels sufficiently elevated to drive cell proliferation and is also increased following respiratory virus infection. While it is generally assumed that these changes are associated with tissue repair following inflammation, our
data implicate thrombin-mediated signaling as both an innate strategic initiator and an effector-
perpetuator of allergic sensitisation through its direct generation by inhaled Der p 1.

That the TLR-3 ligand poly i:c operates ROS generation through a mechanism which converges with
Der p 1 signaling at pannexons is interesting because interactions between allergens and respiratory
viruses precipitate exacerbations of asthma and allergy-polarizing transcription factors are redox
sensitive. PAR1 contributes to the pathogenicity of influenza A, PAR1 and TLR3 are both up-
regulated by respiratory virus infections, ATP promotes Th2 immunity, and P2X7 expression is up-
regulated in asthma. It will therefore be of interest to investigate the operational role of pannexons
as a signaling nexus in allergic sensitisation and the triggering of disease exacerbations.

The sensitivity of TLR3-mediated activation to argatroban or PAR1 antagonists (see Fig E2e-f in this
article’s online repository at www.jacionline.org) suggests that events downstream of pannexon
opening involve the endogenous activation of thrombin, creating a cyclical process. These findings
reveal a surprising primary trigger for thrombin production which further emphasize its contribution
to inflammatory lung responses. While an oral thrombin inhibitor, albeit with bioavailability and
protein binding which may preclude significant airway access from the systemic circulation, has only
moderate improving effect on HDM-induced pathology in a murine model, our data suggest that it
would be of interest to explore similar effects of ADIs, especially as these molecules have been
optimised with the pharmaceutical credentials for inhaled delivery.

Additional information is available (see this article’s Methods, Results and References in the Online
Repository at www.jacionline.org).
Fig 1. A,B, ROS production in calu-3 cells and primary cultures of human bronchial epithelial cells, respectively, following vehicle (veh) or HDM allergen treatment (*P<0.001 v veh control). C,D, MitoSOX red/NucBlue staining of calu-3 cells following veh or HDM. E, Attenuation of HDM induced ROS production by Der p 1 inhibitors (*P<0.001 v veh control; **P<0.001 v HDM). F, Inhibition of Der p 1 by ADZ 51,457 (*P<0.001 v veh control; **P<0.001 v corresponding Der p 1 concentration). G,H, Inhibition of HDM allergen-induced ROS production by the PAR1 antagonist SCH 79797 or by siRNA knockdown (*P<0.001 v veh; **P<0.001 v HDM 1 with or without control (con) transfection. I, ROS production by HDM allergens or by poly i:c is reduced in cells following knockdown of pannexin 1 (*P<0.001 v veh; **P<0.001 v HDM 1 with or without control transfection; †P<0.05 v poly i:c; ‡P<0.001 v poly i:c with or without control transfection).
Fig 2. A, B, AZ 10606120 inhibits ROS production by HDM allergens or poly i:c (*P<0.001 v veh; **P<0.001 v HDM or poly i:c, respectively). C, D, Argatroban inhibits ROS generation by HDM allergens and by poly i:c (*P<0.001 v veh; **P<0.001 v HDM or poly i:c. F, Antagonism of poly i:c-dependent ROS production by PAR1 antagonist FR 171113 (P<0.001 except at 3 µM). G, Time-dependent proteolysis of prothrombin 1 by mixed HDM allergens and its inhibition by ADZ 50,000.
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