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

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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.

1   *To the Editor:*

2   Group 1 cysteine protease allergens from house dust mites (HDMs) are targets of a new class of

3   drugs known as Allergen Delivery Inhibitors (ADIs) which are entering development for asthma

4   therapy.[1] In studying pro-inflammatory signaling by protease allergens, attention has focused on

5   their direct cleavage of protease-activated receptors (PARs).[2] We have explored an alternative view,

6   namely that a key HDM allergen triggers the activation of thrombin and stimulates production of

7   intracellular reactive oxygen species (ROS) and the extracellular release of ATP. ROS have

8   significance because they orchestrate an allergic polarization of immune responses, and both

9   heightened ROS production and a broad deficit in anti-oxidant defenses are characteristics of

10   asthma.[3]

11

12   To investigate the production of intracellular ROS we loaded human airway epithelial cells (primary

13   cultures and established lines) with dihydrorhodamine 123 and exposed them to a natural mixture

14   of *D. pteronyssinus* allergens. This resulted in a sustained generation of ROS (**Fig 1A,B**) associated

15   with mitochondria and nuclei (see **Methods** and **Fig 1C,D** and **Fig E1a-d** in this article's online

16   repository at www.jacionline.org).

17

18   ADZ 51,457 and ADZ 51,529, which are reversible ADIs targeting Group 1 HDM protease allergens[1],

19   substantially reduced ROS generation (**Fig 1E**). Purified natural Der p 1 replicated ROS production

20   and was fully inhibited by ADZ 51,457 (**Fig 1F**). In contrast, an irreversible inhibitor of serine

21   proteases had no effect on ROS production and purified Der p 2 conspicuously failed to elicit ROS

22   generation (**Fig E2a,b** in this article's online repository at www.jacionline.org). Thus, among natural

23   HDM allergens the initiators of intracellular ROS generation are the Group 1 cysteine proteases.

24

25   Surprisingly, ROS production by HDM allergens was transduced through PAR1 and PAR4, with only a

26   small contribution from PAR2 (**Fig 1G,H**, and **Fig E3a-e** in this article's online repository at

27   www.jacionline.org). These responses required the opening of pannexons which are *inter alia*

28   conduits for ATP release (**Fig 1I**). Interestingly, the viral RNA surrogate polyinosinic:polycytidylic acid

29   (poly i:c) also caused pannexon-dependent ROS production (**Fig 1I**). Although HDM allergens and

30   poly i:c initiated ROS production differently (**Fig E4a,b** in this article's online repository at

31   www.jacionline.org), their signaling converges at pannexons (**Fig. 1I**) with the extracellular release of

32   ATP and activation of mechanisms sensitive to the allosteric P_2X_7 receptor modulator, AZ 10606120

33   (**Fig 2A,B**).

34

35   Stimulation of PAR1 and PAR4 has not previously been associated with Der p 1^2, so we were

36   interested in determining whether this involved the generation of thrombin, their canonical

37   activator. The thrombin inhibitor argatroban inhibited ROS generation by HDM allergens, whereas

38   the Factor Xa inhibitor apixaban was without effect (**Fig 2C,D** and **Fig E5a-c** in this article's online

39   repository at www.jacionline.org), thus excluding thrombin formation by the full coagulation

40   cascade. Interestingly, both argatroban and a PAR1 antagonist were effective inhibitors of poly i:c

41   (**Fig 2E,F**).

42

43   Incubation of prothrombin with mixed HDM allergens caused the appearance of prethrombin- 1, the

44   zymogen form of meizothrombin desF1, and the B chain of thrombin as major products. This process

45   was inhibited by ADZ 50,000, an irreversible active site titrant analogue of ADZ 51,457 and ADZ

46    $51,529^1$ (**Fig 2G**). Formation of thrombin by Der p 1 provides further insight into the PAR siRNA data

47   (**Fig 1G,H**) and a possible explanation of the extensive antagonism of ROS formation by PAR1

48   antagonists (SCH 79797, FR 171113) and the PAR4 antagonist, tcY-NH$_2$ (see **Fig E3a-c** in this article's

49   online repository at www.jacionline.org). Heterodimerization of PAR1 and PAR4 is precedented,

50   providing a mechanism for thrombin bound to PAR1 through exosite 1 to cleave PAR4 (which cannot

51   bind) more efficiently.[4] The formation of a ternary complex would thus render ROS generation

52   sensitive to antagonism of both receptors and imply that the main effector of Der p 1-stimulated

53   ROS production might be PAR4, which is notably associated with epithelial-mesenchymal transition

54   in airway cells.

55

56   Hitherto, PAR1 and PAR4 have not been considered activatable by Group 1 HDM allergens[2], but in

57   revealing the Der p 1-dependent cleavage of prothrombin we have identified their canonical

58   activation with subsequent intracellular ROS formation via ATP release. Extracellular ATP is elevated

59   in asthma, which is noteworthy because it stimulates dendritic cells and triggers the release of IL-33,

60   which is genetically linked to asthma susceptibility and a key activator of cytokine production by iH$_2$

61   nuocytes[5]. Thrombin is present in airway surface liquid in asthma at levels sufficiently elevated to

62   drive cell proliferation and is also increased following respiratory virus infection [6]. While it is

63   generally assumed that these changes are associated with tissue repair following inflammation, our

64   data implicate thrombin-mediated signaling as both an innate strategic initiator and an effector-

65   perpetuator of allergic sensitisation through its direct generation by inhaled Der p 1.

66

67   That the TLR-3 ligand poly i:c operates ROS generation through a mechanism which converges with

68   Der p 1 signaling at pannexons is interesting because interactions between allergens and respiratory

69   viruses precipitate exacerbations of asthma and allergy-polarizing transcription factors are redox

70   sensitive. PAR1 contributes to the pathogenicity of influenza A[7], PAR1 and TLR3 are both up-

71   regulated by respiratory virus infections[8], ATP promotes Th_2 immunity, and P_2X_7 expression is up-

72   regulated in asthma.[5] It will therefore be of interest to investigate the operational role of pannexons

73   as a signaling nexus in allergic sensitisation and the triggering of disease exacerbations.

74

75   The sensitivity of TLR3-mediated activation to argatroban or PAR1 antagonists (see **Fig E2e-f** in this

76   article's online repository at www.jacionline.org) suggests that events downstream of pannexon

77   opening involve the endogenous activation of thrombin, creating a cyclical process. These findings

78   reveal a surprising primary trigger for thrombin production which further emphasize its contribution

79   to inflammatory lung responses. While an oral thrombin inhibitor, albeit with bioavailability and

80   protein binding which may preclude significant airway access from the systemic circulation, has only

81   moderate improving effect on HDM-induced pathology in a murine model[9], our data suggest that it

82   would be of interest to explore similar effects of ADIs, especially as these molecules have been

83   optimised with the pharmaceutical credentials for inhaled delivery.

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85   Additional information is available (see this article's Methods, Results and References in the Online

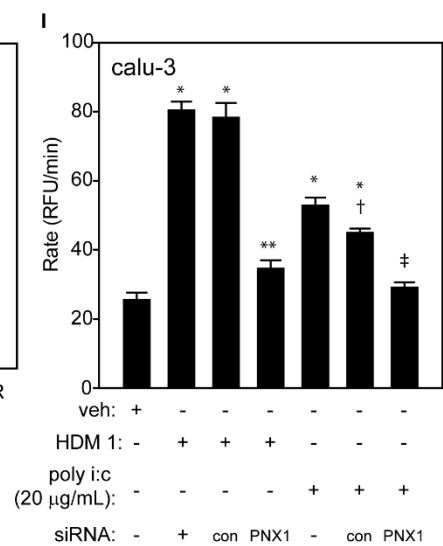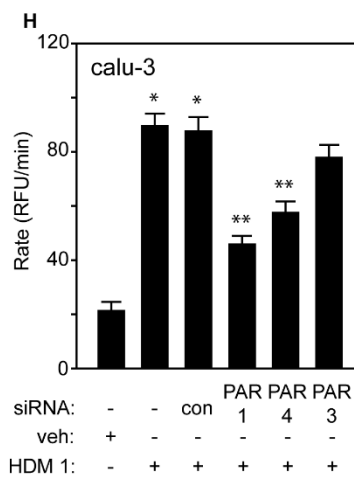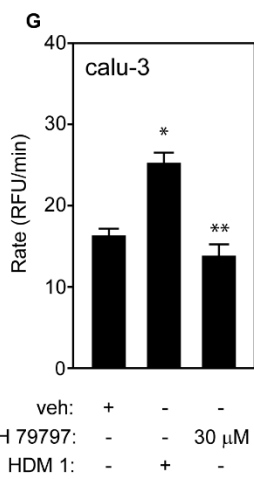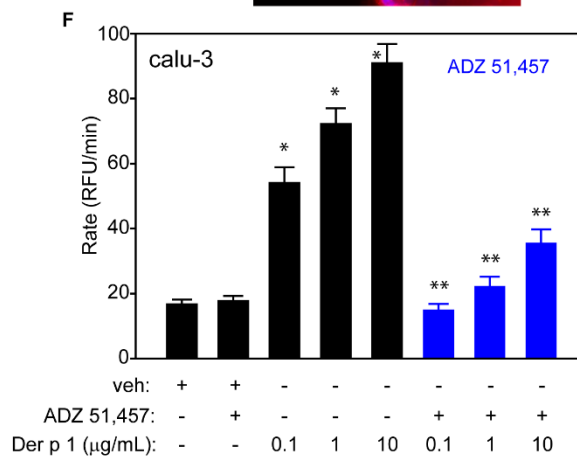86   Repository at www.jacionline.org).
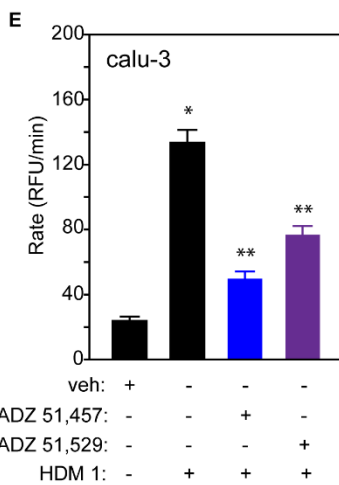
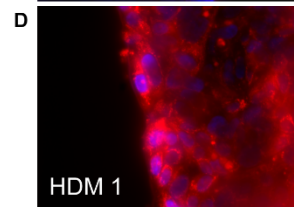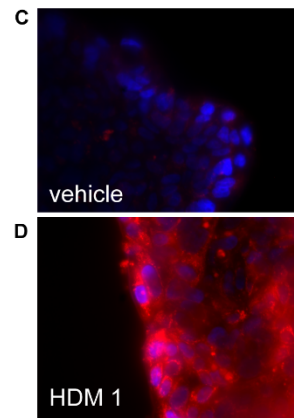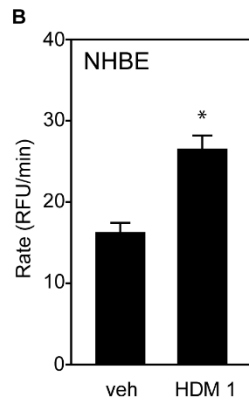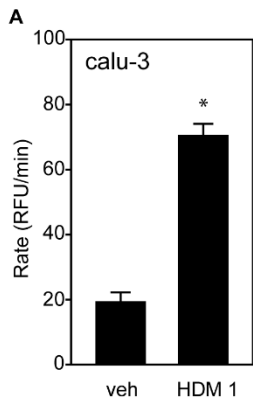**Fig 1. A,B,** ROS production in calu-3 cells and primary cultures of human bronchial epithelial cells, respectively, following vehicle (veh) or HDM allergen treatment (*P<0.001 *v* veh control). **C,D**, MitoSOX red/NucBlue staining of calu-3 cells following veh or HDM1. **E,** Attenuation of HDM induced 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).

A

calu-3

veh:	+	-	-	+	-	-
AZ 10606120 (nM):	-	-	-	50	50	50
HDM:	-	0.1	1	-	0.1	1

B

calu-3

veh:	+	-	-	-
poly i:c (20 μg/mL):	-	+	+	+
AZ 10606120 (nM):	-	-	50	500

C

calu-3

D

NHBE

veh:	+	-	-
argatroban:	-	-	30 μM
HDM 1:	-	+	+

E

calu-3

veh:	+	-	-
argatroban:	-	-	10 μM
poly i:c (20 μg/mL):	-	+	+

F

calu-3

G

| Incubation time (min) | | HDM | | | | | | | HDM + ADZ 50,000 | | | | | |
| | 5 | 10 | 20 | 40 | 80 | 120 | 180 | 5 | 10 | 20 | 40 | 80 | 120 | 180 |

prothrombin

prethrombin-1

thrombin

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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