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A critical role for LTA₄H in limiting chronic pulmonary neutrophilic inflammation

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Abstract

Leukotriene A₄ Hydrolase (LTA₄H) is a pro-inflammatory enzyme which generates the inflammatory mediator leukotriene B₄ (LTB₄). LTA₄H also possesses aminopeptidase activity with unknown substrate and physiological significance. We identified the neutrophil chemoattractant, Pro-Gly-Pro (PGP), as this physiological substrate. PGP is a biomarker for chronic obstructive pulmonary disease (COPD), and is implicated in neutrophil persistence in the lung. In acute neutrophil driven inflammation, PGP was degraded by LTA₄H, which facilitated the resolution of inflammation. In contrast, cigarette smoke, a major risk factor for the development of COPD, selectively inhibited LTA₄H aminopeptidase activity, which led to the accumulation of PGP and neutrophils. These studies imply that therapeutic strategies that inhibit LTA₄H to prevent LTB₄ generation may not reduce neutrophil recruitment because of elevated PGP.

Neutrophils are a critical component of the host's defense against microorganisms (1,2); however, cessation of neutrophil recruitment and clearance by apoptosis is mandatory to

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Supporting Online Material

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Materials and methods

Supporting online text

Figs. S1, S2, S3, S4, S5, S6, S7, S8, S9, S10

Tables S1, S2

restore homeostasis and limit host tissue damage (3,4). Chronic neutrophilic inflammation is observed in lung diseases such as cystic fibrosis (CF) and COPD, mediating extensive tissue damage and contributing to organ dysfunction (5,6). Classical chemoattractants for neutrophils are the Glutamic acid-Leucine-Arginine (ELR⁺) motif containing CXC chemokines (7), such as interleukin (IL)-8 (CXCL8) in humans and KC (CXCL1) and MIP-2 (CXCL2) in mice. Proline-Glycine-Proline (PGP) is a tripeptide generated from the breakdown of extracellular matrix (ECM) collagen and is specifically chemotactic for neutrophils *in vitro* and *in vivo* (8,9). N-terminal acetylation of PGP (AcPGP), which occurs through an unknown mechanism, can enhance its chemotactic potential (10). PGP/AcPGP share sequence homology to key motifs found in the majority of ELR⁺ CXC chemokines and bind to their receptors, CXCR1 and CXCR2 (8,9). PGP is generated from native collagen by the action of matrix metalloproteinase-8 (MMP-8) and/or MMP-9 followed by a secondary cleavage by prolyl endopeptidase (PE) (9). We have subsequently identified significant quantities of PGP or AcPGP in clinical samples from patients with chronic lung diseases such as CF, COPD and bronchiolitis obliterans syndrome (BOS) (8,9,11,12), where it functions to promote the maintenance of neutrophilic inflammation at a time of declining classical chemokine levels. Here we investigate the role of PGP in acute pulmonary neutrophilic inflammation.

Influenza infection of mice elicits an acute neutrophilic inflammation peaking at 24 hours post infection (13) (fig. S1, A and B), coinciding with peak KC and MIP-2 levels (fig. S1, C and D). MMP-9 protein expression and activity were elevated at 24 hours post infection and persisted for several days, before subsiding to baseline levels (fig. S1, E–G). PE activity (fig. S1H) and protein amounts (fig. S1I) were also rapidly elevated in BALF following influenza infection, peaking at 24 hours after infection. Thus the entire enzyme repertoire required for PGP generation was present. Neutrophils were demonstrated to be a prominent source of both MMP-9 and PE (13) (fig. S1, J and K). Despite the presence of MMP-9 and PE, however, no PGP was detectable at any time point analyzed (fig. S2A) utilizing a highly sensitive mass spectrometry technique (8).

Absence of PGP during influenza infection led us to question whether an enzyme was present that degraded it. BALF from 24 hours post influenza infection (maximal MMP-9/PE) exhibited a potent capacity to degrade exogenous PGP, as determined by mass spectrometry (Fig. 1A). Generation of free proline, from PGP degradation, by influenza BALF correlated with loss of PGP by mass spectrometry (Fig. 1B), with one proline being cleaved per PGP molecule. N-terminal acetylation of PGP protected it from degradation (fig. S2B). BALF from different time points post influenza infection was subsequently incubated with PGP and degradation was assessed by mass spectrometry and free proline generation (Fig. 1C). Naïve BALF had the capacity to degrade PGP, but activity was markedly increased after influenza infection, peaking 24–48 hours, before subsiding to basal activity. Lung epithelial cells and neutrophils were shown to be prominent sources of this PGP degrading activity (13) (fig. S2, C–E). Thus both PGP generating and degrading enzymes are similarly up-regulated during influenza infection coinciding with neutrophilic inflammation.

The enzyme responsible for degrading PGP was isolated from influenza BALF and demonstrated to be leukotriene A₄ hydrolase (LTA₄H) (13) (fig. S3 and table S1). LTA₄H acts as an epoxide hydrolase to catalyze the conversion of LTA₄ to the pro-inflammatory mediator LTB₄ (14,15). It has also been suggested to have aminopeptidase capacity, but no physiological substrate has been identified (16,17). We incubated recombinant human LTA₄H with PGP with and without bovine serum albumin (BSA) because its aminopeptidase activity is reportedly enhanced by albumin (18), and assessed PGP degradation (Fig. 2, A and B). LTA₄H had a potent capacity to degrade PGP which was

augmented at lower enzyme concentrations by BSA. Recombinant LTA₄H did not degrade AcPGP (fig. S4A). LTA₄H displayed a significantly greater K_{cat} for a PGP substrate than for LTA₄, but also possessed a greater K_m. Consequently, the specific activity (K_{cat}/K_m) of LTA₄H for PGP was approximately $1 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$, which compares favorably with reported specific activities for LTA₄H with LTA₄ (13) (fig. S4, B–D and table S2) (17). Murine recombinant LTA₄H was expressed and purified (fig. S5, A–C) and demonstrated to possess a comparable capacity to degrade PGP (fig. S5, D–G).

LTA₄H selectively cleaved the N-terminal amino acid from tripeptides and was inhibited by tripeptide aminopeptidase inhibitor bestatin but not other general aminopeptidase inhibitors such as amastatin (13) (fig. S6, A and B). Biological samples containing MMP-9/PE can generate PGP from collagen *ex vivo* (9). Although BALF incubated with collagen failed to generate PGP, co-incubation with bestatin yielded small quantities of PGP with naive BALF and substantial quantities with BALF from 24 hours post influenza infection (Fig. 2C and fig. S2C). Bestatin or amastatin were subsequently administered intratracheally to influenza-infected mice. Although we did not detect PGP in the BALF from untreated mice at 24 hours post infection, those administered bestatin generated significant concentrations of PGP in their BALF (Fig. 2D) and possessed significantly greater numbers of neutrophils in their lung tissue (Fig. 2E). It would therefore appear that LTA₄H degrades collagen derived PGP, limiting neutrophilic inflammation.

To verify a role for LTA₄H in PGP degradation *in vivo*, we next assessed the amount of PGP in the BALF of wild type (13) (fig. S7, A–D) and *Lta4h*^{−/−} mice. Naive wild type mice possessed no PGP, whereas *Lta4h*^{−/−} mice exhibited low but reproducible concentrations of PGP (Fig. 3A). This phenotype was more pronounced at day 3 post-influenza infection (peak neutrophil infiltrate) (Fig. 3B). *Lta4h*^{−/−} mice failed to produce LTB₄, but also possessed lower amounts of KC and MIP-2 in BALF relative to wild types (Fig. 3, A and B). There was a small yet significant increase in neutrophil numbers in the lungs of *Lta4h*^{−/−} mice at day 3 of influenza infection (Fig. 3C); however the contribution of PGP may be partially masked because of the reduced amounts of other chemoattractants. To address this, we measured neutrophil chemotaxis *ex vivo* to wild type and *Lta4h*^{−/−} BALF (day 3 post-influenza infection) in the presence of a PGP neutralizing antibody. BALF from both groups of mice was chemotactic for neutrophils and chemotaxis was suppressed by neutralization of KC/MIP-2 (fig. S8A). Neutralization of PGP, however, caused a marked reduction in the chemotactic potential of *Lta4h*^{−/−} BALF only (Fig. 3D). The BALF of wild type mice could degrade exogenous PGP, which was markedly increased with influenza infection; however, degradation was absent with BALF from *Lta4h*^{−/−} mice (Fig. 3, E and F). Furthermore, whereas BALF from influenza infected wild type mice generated negligible levels of PGP from collagen *ex vivo* (fig. S8, B and C), BALF from *Lta4h*^{−/−} mice generated significant quantities. PGP generation was significantly reduced by co-incubation with inhibitors to MMP-9 and PE, verifying the importance of these enzymes in generation of the tripeptide (fig. S8, B and C).

Failure to detect PGP owing to degradation of the tripeptide was not unique to influenza infection, because comparable results were obtained in response to *Streptococcus pneumonia* (13) (fig. S9). We next questioned why PGP was present in chronic neutrophilic conditions such as COPD (12), because LTA₄H so potently degrades it. Chronic instillation of AcPGP into the lungs of mice results in neutrophil recruitment and an emphysematous lung phenotype (8,19). We demonstrated that cigarette smoke condensate (CSC) could N-terminally acetylate PGP (Fig. 4A). N-terminal acetylation not only protects PGP from degradation by LTA₄H but also enhances its chemotactic capacity (10). Exposure to CSC also dose dependently inhibited the capacity of LTA₄H to degrade PGP (Fig. 4, B and C). CSC appeared to exhibit selective inhibition of the aminopeptidase activity of LTA₄H, with

minimal inhibition of the hydrolase activity observed (Fig. 4D). Furthermore, no difference was observed in LTB₄ generation in response to the chemoattractant, formyl-met-leu-phe (fMLP), when neutrophils were in the presence of CSC (fig. S10). Selective inhibition of aminopeptidase activity is feasible because residues have been delineated that are critical to one activity but have no role in the other (20,21), and selective modulation has previously been observed in response to biological and chemical mediators (18,22,23).

We have previously demonstrated that lung epithelial cells release LTA₄H (fig. S2C). Exposure of primary human bronchial epithelial cells to a non-lethal dose of CSC significantly reduced the aminopeptidase activity of the released LTA₄H (Fig. 4, E and F), whereas substantially increasing the detectable amount of total LTA₄H protein released (Fig. 4G). It is important to note that, in this instance, whilst the apparent percentage inhibition of activity is less than 30 %, the amount of LTA₄H protein released by the CSC stimulated epithelial cells is well in excess of that released by control treated cells and so the actual inhibition is far greater. Indeed, the increase in total LTA₄H protein with CSC is in excess of 10-fold and thus the actual relative inhibition is likely to be in excess of 95%. Furthermore, single exposure of mice to intranasal cigarette smoke extract markedly reduced the capacity of BALF (24 hours later) to degrade PGP *ex vivo* (Fig. 4, H and I) and led to significant concentrations of AcPGP in BALF (Fig. 4J) and neutrophil recruitment (Fig. 4K) *in vivo*.

In this study, we propose disparate functions of LTA₄H in generating one neutrophil chemoattractant (LTB₄), whilst degrading another (PGP). LTA₄H is generally assumed to be a cytosolic enzyme, but we report an extracellular role, corroborating detection of this enzyme previously in the plasma of various mammals and in human BALF (24,25). The peptidase, but not the epoxide hydrolase, activity of LTA₄H is augmented in the presence of albumin (18) and chloride ions (22). The disparate concentrations of Cl⁻ and albumin intracellularly and extracellularly, support the notion that the epoxide hydrolase activity of LTA₄H operates intracellularly and the aminopeptidase capacity occurs extracellularly. Neutrophils are a source of enzymes that both generate and degrade PGP, and are therefore capable of promoting and then restricting their own recruitment.

Our results also suggest that cigarette smoke shifts the emphasis of LTA₄H, which has dual pro-inflammatory and anti-inflammatory functions towards a pro-inflammatory phenotype. Consequently, a combination of LTB₄ and PGP, both implicated in the pathogenesis of COPD (8,12,19,26), could drive the observed inflammation. Such selective modulation of the disparate activities of LTA₄H may not be unique to COPD. Extracellular levels of chloride ions, which selectively activate the aminopeptidase activity, are diminished in CF patients owing to defective Cystic fibrosis transmembrane conductance regulator (CFTR) (27) and could explain the PGP observed in these patients (9).

Finally, LTB₄ is implicated in acute and chronic inflammatory diseases (28–31). The development of drugs that seek to inhibit LTA₄H activity and LTB₄ generation to alleviate pathologies of inflammatory disease are underway. When developing these drugs it will be critical to consider the potential repercussions of preventing PGP degradation.

Supplementary Material

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Acknowledgments

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Fig. 1.

Influenza infection induces the release of PGP degrading enzymes. **(A and B)** Undiluted, diluted 1/10 or 1/100 BALF from 24 hours after influenza infection was incubated with PGP and degradation assessed by mass spectrometry **(A)**, expressed as percentage degradation relative to peptide alone, or **(B)** free proline generated. **(C)** 1/10 diluted BALF from indicated times post-influenza infection was incubated with PGP and degradation was assessed after 2 hours by mass spectrometry or free proline generation. Data (mean $\pm$ SD, **A and B**; mean $\pm$ S.E.M., **C**) are representative of three experiments with triplicates **(A and B)** or four mice per group **(C)**.

Fig. 2. Leukotriene A₄ Hydrolase degrades PGP. (**A and B**) Degradation of PGP by 1 µg/ml, 0.1 µg/ml and 0.01 µg/ml recombinant human LTA₄H with or without BSA, as determined by (**A**) mass spectrometry or (**B**) generation of free proline. (**C**) Generation of PGP from type II collagen by BALF from naive and influenza infected (24 hours) mice, with or without bestatin. (**D**) PGP in BALF and (**E**) neutrophil numbers in lung tissue 24 hours after influenza infection in mice administered intratracheal PBS (UT), bestatin (Best.) or amastatin (Amast.). *, P<0.05, **, P<0.01. Data (mean ± SD **A–C**; mean ± S.E.M., **D and E**) are representative of three experiments with triplicates (**A–C**) or four mice per group (**D and E**).

Fig. 3. *Lta4h*^{-/-} mice lose capacity to degrade PGP. The concentrations of KC, MIP-2, LTB₄ and PGP in BALF of (A) naive or (B) influenza infected (day 3) wild type and *Lta4h*^{-/-} mice. (C) Neutrophil numbers in the lungs of naive or influenza infected (day 3) wild type or *Lta4h*^{-/-} mice. (D) Inhibition of wild type and *Lta4h*^{-/-} BALF induced neutrophil chemotaxis *ex vivo* by PGP neutralization. (E and F) BALF, diluted 1/10, from naive or influenza infected (day 3) wild type and *Lta4h*^{-/-} mice incubated with PGP and degradation assessed after 2 hours by (E) mass spectrometry or (F) free proline generation. *, P<0.05, **, P<0.01. Data (mean ± S.E.M., A–C; mean ± SD, D–F) from four mice per group (A–C) or triplicates (D–F).

Fig. 4. Cigarette smoke inhibits PGP degradation by LTA₄H. **(A)** Acetylation of PGP by 0.5 %, 1 %, 2 % and 4 % CSC or vehicle control (2% DMSO). **(B and C)** Degradation of PGP by LTA₄H alone or in presence of 0.5 %, 1 %, or 2% CSC or vehicle control, as determined by **(B)** mass spectrometry or **(C)** generation of free proline. **(D)** Inhibition of hydrolase and aminopeptidase activities by 2% CSC. **(E–G)** Human lung epithelial cells cultured with 2 % CSC or vehicle control for 24 hours. **(E and F)** Degradation of PGP after 2 hours by apical supernatant (diluted 1/10), as assessed by **(E)** mass spectrometry or **(F)** free proline generation. **(G)** Western blot for LTA₄H in apical supernatant from 2 % CSC or vehicle control treated cells. Each lane represents apical supernatant from a different experiment. **(H and I)** BALF, diluted 1/10, from vehicle control or cigarette smoke extract (CSE) treated mice (24 hours post treatment) incubated with PGP and degradation assessed after 2 hours by **(H)** mass spectrometry or **(I)** free proline generation. AcPGP **(J)** and neutrophil numbers **(K)** in BALF of mice 24 hours post administration of vehicle control or CSE. *, $P < 0.05$, **, $P < 0.01$. Data (mean $\pm$ SD, **A–F**; mean $\pm$ S.E.M., **H–K**) are representative of at least triplicates (**A–F**) or three experiments with four mice per group (**H–K**).