Pneumolysin potentiates oxidative inactivation of alpha-1-proteinase inhibitor by activated human neutrophils

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Summary This study was designed to investigate the effects of the \textit{Streptococcus pneumoniae}-derived, pro-inflammatory toxin, pneumolysin (8.37 and 41.75 ng/ml), on the oxidative inactivation of alpha-1-protease inhibitor (API) by chemoattractant-activated human neutrophils in vitro. The elastase inhibitory capacity (EIC) of API in supernatants from unstimulated neutrophils, neutrophils treated with pneumolysin only, or with the chemoattractant FMLP (1 \textmu M) only, or the combination of the toxin with FMLP was measured by a colorimetric procedure based on the activity of added porcine elastase. The EIC of API was unaffected by exposure to pneumolysin only, unstimulated neutrophils, or neutrophils treated with pneumolysin only. However, exposure to FMLP-activated neutrophils resulted in a reduction of the EIC of API, which was significantly augmented by pneumolysin (mean reductions of 16%, 43% and 83% for FMLP only and in combination with 8.37 and 41.75 ng/ml pneumolysin, respectively), and was attenuated by wortmannin (1 \textmu M), an inhibitor of NADPH oxidase, the oxidant-scavenger methionine (100 \textmu M), and depletion of Ca\textsuperscript{2+} from the cell-suspending medium. These pro-proteolytic interactions of pneumolysin with chemoattractant-activated neutrophils may contribute to the invasiveness of the pneumococcus.

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Introduction

Pneumolysin, a thiol-activated polypeptide, is one of the best characterized virulence factors produced by the pneumococcus, possessing cytotoxic and pro-inflammatory properties, both of which are believed to contribute to the pathogenesis of invasive disease.\textsuperscript{1-5} Notwithstanding the complement-activating properties of pneumolysin, the pro-inflammatory actions of the toxin are secondary to plasma membrane modifications in cells such as neutrophils and monocytes which cause influx of
Ca$^{2+}$ and augmentation of the production of reactive oxidants, prostanoids, eicosanoids, cytokines and chemokines both in vitro and in vivo,\textsuperscript{1,2,6,7} all of which amplify neutrophil recruitment to the airways.\textsuperscript{8} However, rather than eradicating the infection, this over-exuberant inflammatory response appears to favor persistence and extrapulmonary dissemination of the pneumococcus, possibly as a consequence of damage to airway epithelium mediated directly by pneumolysin, as well as by reactive oxidants and proteases released by pneumolysin-sensitized neutrophils.\textsuperscript{9–12} In addition to these mechanisms, the onslaught directed against airway epithelium may be intensified by hydrogen peroxide released in copious amounts by the pneumococcus.\textsuperscript{13}

There is a growing body of evidence that alpha-1-protease inhibitor (API), a primary, albeit oxidation-sensitive, inhibitor of neutrophil elastase is critically involved in pulmonary host defenses, protecting the lungs not only against inflammatory insults,\textsuperscript{14} but possibly against microbial pathogens.\textsuperscript{15,16} This contention is supported by several clinical studies which have reported that API undergoes functional inactivation, both oxidative and proteolytic, in the lungs of patients with acute pneumonia, the magnitude of which appears to be a determinant of unfavorable outcome.\textsuperscript{17–22}

Surprisingly, given the apparent importance of API in pulmonary host defense, the interactions between pneumolysin, neutrophils and API have not been described. To address this important topic, the current study has focused on the effects of pneumolysin alone and in combination with activated neutrophils on the elastase inhibitory capacity of API.

**Materials and methods**

**Preparation of pneumolysin**

Recombinant pneumolysin was expressed in *Escherichia coli* and purified from cell extracts as previously described.\textsuperscript{23} Protein homogeneity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The stock protein concentration was 0.21 mg/ml, which corresponds to 1.3 x 10$^6$ hemolytic units/ml, and the stock was essentially free of contaminating bacterial endotoxin (<2 pg/ml). The toxin was diluted in Hanks’ balanced salt solution (HBSS [pH 7.4]; 1.25 mM CaCl$_2$; Highveld Biological Pty Ltd, Johannesburg, South Africa) and used at fixed, final concentrations of 8.37 and 41.75 ng/ml which we have previously found to sensitize neutrophils for increased production of reactive oxidants in vitro.\textsuperscript{6}

**Chemicals and reagents**

Unless indicated all other chemicals and reagents were obtained from the Sigma Chemical Co, St. Louis, MO, USA.

**Neutrophils**

Purified neutrophils were prepared from the heparinized (5 U of preservative-free heparin/ml) venous blood of healthy, non-smoking, medication-free adult, human volunteers of average age 40 ± 2 year and separated from mononuclear leukocytes on Histopaque-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatin to remove most of the erythrocytes. After centrifugation residual erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%) determined by light microscopy and fluorescence microscopy (exclusion of ethidium bromide) respectively, were resuspended to 1 x 10$^7$/ml in PBS and held on ice until used. We have previously reported that removal of residual contaminating cells (B- and T-lymphocytes, monocytes and natural killer cells) by flow cytometric procedures did not significantly affect the pro-inflammatory interactions of pneumolysin with neutrophils, while these were completely eliminated by depletion of neutrophils.\textsuperscript{1}

**Elastase inhibitory capacity (EIC) of alpha-1-protease inhibitor (API)**

Purified API (0.25 mg/ml, final) was added to (i) HBSS only (ii) neutrophils (5 x 10$^6$) only (iii) neutrophils + pneumolysin (8.37 and 41.75 ng/ml) (iv) neutrophils + the synthetic chemotaxtractant, N-formyl-$\text{L}$-methionyl-$\text{L}$-leucyl-$\text{L}$-phenylalanine (FMLP, 1 $\mu$M final) or (v) neutrophils + pneumolysin + FMLP. Neutrophils were preincubated with API for 5 min at 37°C prior to the addition of pneumolysin or an equal volume of HBSS to control systems and a further 5 min incubation at 37°C. This was followed by addition of FMLP or an equal volume of HBSS to control systems. The final volume in each tube was 250 $\mu$l and the tubes were then incubated for 15 min at 37°C after which the neutrophils were pelleted by centrifugation and the supernatants
harvested and assayed for EIC using a micro-
modiﬁcation of a spectrophotometric procedure.24
In a preliminary series of experiments it was
established that at this concentration the API
preparation used did not interfere with either the
hemolytic activity of pneumolysin (41.75 ng/ml) or
with the toxin-mediated inﬂux of Ca\(^{2+}\) into
neutrophils.6,25

Briefly, 50 \(\mu\)l of the supernatant were added to
250 \(\mu\)l of 0.2 M Tris–HCl (pH 8.0) containing 21
milliunits (ﬁnal) of porcine elastase and the
mixtures incubated for 15 min at 37 ^\(\circ\)C. Following
incubation, 50 \(\mu\)l of the mixture was added to 150 \(\mu\)l
of the elastase substrate \(N\)-succinyl-L-alanyl-L-
alanyl-L-alanine-p-nitroanalide (0.6 mM in Tris–
HCl) in the wells of microtiter plates and elastase
activity monitored spectrophotometrically at a
wavelength of 405 nm over a 30 min time course
at 37 ^\(\circ\)C. The results are expressed as the magnitude
(\%) of inactivation of the EIC of API.

In an additional series of experiments the effects
of inhibition of neutrophil NADPH oxidase by
wortmannin (selective inhibitor of phosphatidyli-
ositol-3-kinase, 1 \(\mu\)M ﬁnal), as well as the reactiv-
ity of neutrophil-derived hypochlorous acid by
methionine (reactive oxidant scavenger , 100
\(\mu\)M), on neutrophil-mediated inactivation of API were
investigated. These agents were added to the cells
5 min prior to pneumolysin (8.37 ng/ml).

The effects of depletion of extracellular Ca\(^{2+}\)
were investigated by using cells suspended in
nominally Ca\(^{2+}\)-free HBSS containing the Ca\(^{2+}\)-
chelating agent, ethylene glycol-bis (\(\beta\)-aminoethyl
ether)-\(N,N,N',N'\)-tetraacetic acid (EGTA, 100 \(\mu\)M
ﬁnal) to ensure total depletion of the cation. Under
these conditions we were unable to detect inﬂux of
Ca\(^{2+}\) into pneumolysin-treated neutrophils (not shown).

Superoxide production
The effects of pneumolysin (8.37 ng/ml) ± FMLP
(1 \(\mu\)M) in the presence and absence of wortmannin
and methionine on superoxide production by
neutrophils were measured using a lucigenin (bis-
\(N\)-methylacridinium nitrate)-enhanced chemilumi-
nescence (LECL) procedure.6 The incubation condi-
tions were as described above. LECL was monitored over a 5 min time course at 37 ^\(\circ\)C using a
Lumac Biocounter (Lumac Systems) following the
addition of pneumolysin ± FMLP to the cells. The
ﬁnal volume in each vial was 1 ml HBSS supple-
mented with 0.1% bovine serum albumin, contain-
ing 0.2 mM lucigenin and 1 \(\times\) 10\(^6\) neutrophils. The
results are expressed in relative light units (rlu).

Statistical analysis
The primary objective of the study was to
determine the effects of pneumolysin alone and
in combination with FMLP on neutrophil-mediated
oxidative inactivation of the EIC of API. Because of
the uneven distribution of the data, levels of
statistical signiﬁcance were calculated by the
Mann–Whitney U-test (2-tailed) using GraphPad
Instat™ software. All signiﬁcance levels were set
to 5%. The results of each series of experiments are
expressed as the median values with 25–75-
percentiles.

Results
Pneumolysin and API
As shown in Fig. 1, the EIC of API was unaffected by
pneumolysin alone (at 8.37 and 41.75 ng/ml),
neutrophils alone, or the combination of pneumo-
lysin with neutrophils. However, addition of FMLP
(1 \(\mu\)M) to neutrophils was accompanied by signiﬁ-

![Figure 1](image-url)
cant (\(P<0.05\)) inhibition of the EIC of API which was substantially augmented in a dose-related manner by pre-treatment of the cells with pneumolysin (\(P<0.05\) for comparison between FMLP only and FMLP + pneumolysin at both concentrations tested, as well as for comparison between the two concentrations of pneumolysin). The inhibitory effects of FMLP-activated neutrophils in both the absence and presence of pneumolysin on the EIC of API were completely attenuated by depletion of Ca\(^{2+}\) from the cell-suspending medium (Fig. 1).

The effects of treatment of neutrophils with wortmannin and methionine on the inactivation of the EIC of API following activation of the cells with FMLP in the absence and presence of pneumolysin (8.37 and 41.75 ng/ml) are shown in Fig. 2. These agents significantly (\(P<0.05\)) attenuated the functional inactivation of API by FMLP-activated neutrophils both with and without pneumolysin.

### Superoxide generation

The effects of pneumolysin (8.37 ng/ml) on superoxide generation by FMLP-activated neutrophils in the presence and absence of wortmannin (1 \(\mu\)M) and methionine (100 \(\mu\)M) are shown in Fig. 3. As reported previously, pneumolysin per se did not affect the generation of superoxide by resting neutrophils (not shown), but significantly augmented the production of the reactive oxidant by FMLP-activated cells. Inclusion of wortmannin dramatically decreased the production of superoxide by FMLP-activated neutrophils + pneumolysin, while methionine had no detectable effects.

### Discussion

In the current study, pneumolysin, at concentrations which are not cytotoxic over the time course studied, and which are well within the range of

![Figure 2](image1.png)  
**Figure 2** Box and whisker plots of the magnitude of inactivation of the elastase inhibitory capacity (EIC) of alpha-1-protease inhibitor (API) exposed to neutrophils only (control), or to FMLP (1 \(\mu\)M)-activated neutrophils + pneumolysin (Pln, 8.37 and 41.75 ng/ml) in the presence and absence of methionine (100 \(\mu\)M), or wortmannin (1 \(\mu\)M). The data shown are those from 2 different experiments with 5 replicates for each system in each experiment, and are expressed as the median values with 25-75-percentiles. \(P<0.05\) for comparison of the values for FMLP-activated neutrophils in the absence of pneumolysin with the corresponding values in the presence of the toxin.

![Figure 3](image2.png)  
**Figure 3** Box and whisker plots of the effects of methionine (100 \(\mu\)M) and wortmannin (1 \(\mu\)M) on FMLP (1 \(\mu\)M)-activated production of superoxide by neutrophils in the presence of pneumolysin (pnl, 8.37 ng/ml). The data from 2 different experiments with 6 replicates for each system are expressed as the median values in relative light units (rlu, background values subtracted) with 25-75-percentiles. \(P<0.05\) for comparison of the effects of wortmannin relative to those of the corresponding control systems without and with pneumolysin.
those detected in the cerebrospinal fluid of patients with pneumococcal meningitis, has been found to sensitize human neutrophils for exaggerated inactivation of API on exposure of the cells to the chemoattractant, FMLP. These pro-oxidative interactions of pneumolysin with neutrophils appear to be secondary to the pro-oxidative effects of the toxin on the cells since they were associated with augmentation of FMLP-mediated activation of neutrophil membrane-associated oxidative metabolism and attenuated by wortmannin, an inhibitor of activation of NADPH oxidase. Methionine, which has no inhibitory effects on NADPH oxidase, also protected API, presumably by scavenging hypochlorous acid, thereby protecting the critical oxidation-sensitive methionine residue at position 356 of the elastase inhibitory site of API.

The pro-oxidative interactions of pneumolysin with neutrophils are strictly Ca\(^{2+}\)-dependent being secondary to the pore-forming actions of the toxin, which promote influx of extracellular cation and activation or sensitization of Ca\(^{2+}\)-dependent pro-inflammatory functions of the cells. This relationship is also underscored in the current study since depletion of Ca\(^{2+}\) from the cell-suspending medium completely abrogated pneumolysin-mediated enhancement of inactivation of API by FMLP-activated neutrophils.

Because pneumolysin has been reported to augment the release of elastase from FMLP-activated neutrophils, it could be argued that the observed decrease in the EIC of API in this system is simply an artifact due to an excessive amount of elastase (neutrophil-derived enzyme + added porcine elastase). However, we believe that this is unlikely to be the case. This contention is based on the aforementioned observations that the inactivating effects of FMLP/pneumolysin-treated neutrophils on the EIC of API were almost completely abolished by wortmannin and methionine, compatible with a predominantly pro-oxidative mechanism. Nevertheless, pneumolysin-mediated augmentation of neutrophil degranulation may also contribute to free proteolytic activity in the inflamed lung. Because of the short duration (15 min at 37°C) of exposure of neutrophils to pneumolysin in the current study, it seems unlikely that the pro-apoptotic actions of the toxin contribute to its pro-proteolytic activity.

*N*-formylated peptides, of which FMLP is the synthetic prototype, are crucially involved in neutrophil mobilization from the circulation to sites of microbial infection, including pneumococcal infection of the airways. Interestingly, neutrophils from CD38-deficient mice display an impairment of mobilization of intracellular Ca\(^{2+}\) and chemotaxis which is selective for FMLP, and fail to accumulate in the lungs following experimental infection with *Streptococcus pneumoniae*. These observations underscore the relevance of the current in vitro study using FMLP to the pathophysiological setting in pneumococcal disease. By amplifying FMLP-mediated, Ca\(^{2+}\)-dependent signaling, pneumolysin may promote an over-exuberant inflammatory response in the airways, increasing the vulnerability of respiratory epithelium to elastase, which may favor extra-pulmonary dissemination of the pneumoccus. This contention is strengthened by the failure of pneumolysin to potentiate neutrophil pro-inflammatory responses initiated by phorbol esters, which are independent of Ca\(^{2+}\) mobilization by these cells.

Given that API is vulnerable to oxidative, functional inactivation, and that pneumolysin interacts pro-oxidatively with neutrophils, the results of the current study may seem somewhat predictable. Nevertheless, their potential significance with respect to both immunopathogenesis of invasive pneumococcal disease and opportunities for novel interventions, such as the use of synthetic elastase inhibitors, should not be under-estimated. Indeed, this mechanism of augmentation of phagocyte-mediated inactivation of API may also be applicable to other infectious diseases caused by microbial pathogens which, like the pneumococcus, produce pore-forming toxins with pro-oxidative properties.

Interestingly, cigarette smoking has been reported to be an independent risk factor for serious pneumococcal infection, the relative risk being directly related to the extent of the smoking habit. Cigarette smoking also causes inactivation of API in the airways, an effect, which in the light of the observations of the current study, may contribute to the increased risk of invasive pneumococcal disease in smokers. In this setting, the interactive inhibitory effects of exposure to cigarette smoke and pneumolysin may severely compromise the cytotoxic, anti-inflammatory and proposed antimicrobial activities of API in the airways.

It is noteworthy that oxidized API per se possesses proinflammatory properties by promoting neutrophil migration, and activating the production of reactive oxidants, chemokines and cytokines by macrophages, which may further exacerbate inflammatory damage to airway epithelium. Pneumococcal disease is therefore likely to be accompanied by a multi-pronged assault on respiratory epithelium mediated directly by the pneumococcal cytotoxins pneumolysin and hydrogen peroxide.
acting in concert with excessive amounts of phagocyte-derived elastase and reactive oxidants generated by pneumolysin-orchestrated, over-exuberant inflammatory responses.

References


