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Comparative loss of activity of recombinant secretory leukoprotease inhibitor and α_1-protease inhibitor caused by different forms of oxidative stress

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ABSTRACT: Secretory leukoprotease inhibitor (SLPI) and α_1-protease inhibitor (α_1-PI) are powerful antiproteases currently under investigation for their potential to protect the lung from neutrophil elastase (NE). The aim of this study was to determine whether the recombinant form of SLPI (rSLPI) and α_1-PI show different grades of loss of inhibitory activity when exposed to reactive oxygen metabolites.

We incubated rSLPI and α_1-PI with N-chlorosuccinimide (NCS), chloramines, activated polymorphonuclear leucocytes (PMNs) and activated alveolar macrophages (AMs).

Under all conditions evaluated, both antiproteases were partially inactivated. The resulting anti-NE activity of rSLPI was not significantly different from that of α_1-PI after exposure to NCS ($p>0.5$), chloramines ($p>0.6$), activated PMNs ($p>0.07$) and activated AMs ($p>0.9$).

In conclusion, recombinant secretory leukoprotease inhibitor and α_1-protease inhibitor lose antineutrophil elastase activity to a similar extent when exposed to conditions that may be present in inflammatory lung disorders.
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Alpha$_1$-protease inhibitor (α_1-PI) and secretory leukoprotease inhibitor (SLPI) are considered to be the major antiproteases in the human lung. Both molecules are capable of inhibiting a variety of proteases, with neutrophil elastase (NE) likely to be the major target molecule [1–4]. There is evidence of disturbance of the physiological homeostasis between antiproteases and proteases leading to potential unimpeded NE action in several lung disorders, including cystic fibrosis [5], emphysema caused by α_1-PI deficiency [2], emphysema in smokers [6, 7] and adult respiratory distress syndrome (ARDS) [8, 9]. As NE may be destructive, it seems rational to re-establish the physiological balance between proteases and antiproteases by increasing pulmonary protease defences. This therapeutic goal may be achieved by the application of naturally occurring protease inhibitors. Biochemical efficacy of therapy with α_1-PI purified from human plasma [10, 11] and recombinant SLPI (rSLPI) [12, 13] has been demonstrated in patients with inherited α_1-PI deficiency and cystic fibrosis. However, both SLPI and α_1-PI lose activity when exposed to reactive oxygen metabolites. Thus, any difference in the extent of the reduction in anti-NE activity under oxidative stress may be important when considering which antiprotease should be used for therapy.

The aim of our study was to evaluate the consequences of oxidative stress on the anti-NE activity of rSLPI in comparison with α_1-PI. In order to analyse the "pure" oxidant effect, rSLPI and α_1-PI were incubated with a chemical oxidant, N-chlorosuccinimide (NCS) and long-lived naturally occurring oxygen metabolites (chloramines). With the aim to imitate acute pulmonary inflammation (*i.e.* influx of activated polymorphonuclear cells (PMNs) into the lung tissue with the consequence of an increased load of reactive oxygen metabolites and neutrophil proteases) rSLPI and α_1-PI were incubated with activated human PMNs and alveolar macrophages (AMs) obtained from primates activated with the supernatant of stimulated PMNs.

Materials and methods

Recombinant secretory leukoprotease inhibitor and α_1-protease inhibitor

The rSLPI used was a gift from R.C. Thompson (Synergen, Boulder, CO, USA); its synthesis has been described elsewhere [14–16]. The α_1-PI used was a highly purified preparation obtained from human plasma (ART Biochemicals, Athens, GA, USA). The purity

of the preparations of rSLPI and α_1-PI was >99% as shown by sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis and high performance liquid chromatography (HPLC). Given this high grade of purity, the concentrations of rSLPI and α_1-PI in the preparations could be quantified spectrophotometrically (Lambda 16, Perkin Elmer, Überlingen, Germany) based on an extinction coefficient ($E_{1\ cm}$) at 275 nm (0.1% solution) of 0.8 for rSLPI (personal communication by R.C. Thompson) and $E_{1\ cm}$ at 280 nm (1% solution) of 5.3 for α_1-PI [17].

Evaluation of time-independent inhibition of NE by the rSLPI and α_1-PI preparations showed an anti-NE activity of 96±1% and 95±1%, respectively (for methods, see below [18]).

Anti-neutrophil elastase activity

In each of the following experiments, anti-NE activity was quantified using a time-independent titration assay. NE was purified from the sputum of cystic fibrosis patients (Elastin Products, Owensville, MO, USA). The NE activity was quantified in a titration assay with an α_1-PI standard that had been active site titrated with trypsin [18, 19]. Increasing volumes of the sample containing rSLPI or α_1-PI (rSLPI and α_1-PI concentration in final solution 0–2.5 nM) were incubated with a constant volume of the NE standard (active NE concentration in final solution 2 nM). Following an incubation time of 2 h at 23°C, the remaining NE activity was analysed spectrophotometrically (Lambda 16) by addition of the NE-specific synthetic substrate N-methoxy-succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (Sigma, St Louis, MO, USA). Anti-NE activity of the actual sample could be derived from changes in optical density [18, 19] and expressed as a percentage of the anti-NE activity of the initial unmodified rSLPI/α_1-PI preparation (taken as 100%).

N-chlorosuccinimide

The rSLPI and α_1-PI (2.1×10^{-9} mol each) were incubated for 2 h with increasing quantities of NCS (Sigma) at 23°C: molar ratios of the concentration of NCS to the concentration of rSLPI and α_1-PI in the test solution were 0, 0.125, 0.375, 0.5, 0.625, 1.0, 1.5, 3.0 or 6.0. The reaction was stopped by addition of an excess of sodiumthiosulphate (Sigma) 20 µL of a 1 M solution; final volume in each tube 100 µL).

Chloramines

Chloramines were synthesized by addition of taurine (2-aminomethane sulphonic acid; Sigma) to hypochlorous acid (HOCl; Bender und Hobein, Munich, Germany) following the methods of WEISS *et al*. [20] and GRISHAM *et al*. [21]. For the oxidation experiments, the same molar ratios as described above for NCS were chosen.

To ensure that oxidation had occurred under the selected experimental conditions, rSLPI samples treated with NCS or chloramines were evaluated for oxidized methionine residues using the cyanogen bromide method to quantify methionine sulphoxide [22].

Activated polymorphonuclear leucocytes

Peripheral venous blood was obtained from healthy volunteers. PMNs were purified using a variation of the Ficoll hypaque centrifugation method (Polymorphprep®; Nycomed, Oslo, Norway). Using this method, >96% of cells obtained were PMNs. One nanomole each of rSLPI and α_1-PI was added to 0.5×10^6 PMNs in a volume of 400 µL. Cells were stimulated by the addition of 50 µL of a 1 $\mu g \cdot mL^{-1}$ solution of phorbol myristate acetate (PMA; Sigma). The reaction was stopped by introducing test tubes into an ice bath after increasing time intervals (0, 2, 4, 8, 16 and 32 min). Following centrifugation at 4°C, the supernatant was removed and analysed for anti-NE activity after the remaining reactive oxygen metabolites had been inactivated by the addition of methionine in excess (50 µL of a 50 mM solution; Sigma).

To determine whether these experiments were influenced by NE released from PMNs, the amount of NE contained in the PMN supernatant was quantified spectrophotometrically (Lamba 16) by adding N-methoxy-succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (Sigma).

Activated alveolar macrophages

AMs were obtained from monkeys (*Macaca fascicularis*) by bronchoalveolar lavage (BAL) [23]. In the cell differentials, the percentage of AMs was always >90%. Five hundred thousand BAL cells were allowed to adhere to 24-well culture plates (Nunclon®; Nunc, Roskilde, Denmark) for 2 h (37°C, 5% CO_2). Following this, rSLPI or α_1-PI (180 µL, concentration in final solution 2 µM for both) was added. The supernatant of human PMNs (270 µL) that had been stimulated with PMA (for details see above) was incubated with the AMs. In order, principally, to assess the effects of the long-lived oxidants, the cell-free PMN supernatant was kept on ice for 90 min before transfer to the AM solution. The reaction between AMs, PMN supernatant and antiproteases was stopped after varying times by transfer into an ice bath followed by centrifugation at 4°C and addition of excess methionine (see above). The supernatants were again analysed for their anti-NE activity.

To verify that the PMN supernatants used for activation of AMs contained reactive oxygen metabolites, we analysed the "oxidative capacity" of the supernatant. This was achieved by adding pure methionine to the samples (final concentration 500 µM) and by determining the resulting concentration of free methionine sulphoxide residues using reversed-phase liquid chromatography as described elsewhere [22]. In addition, we quantified the myeloperoxidase activity in the PMN supernatants using the method of SUZUKI *et al*. [24].

A series of control-experiments was performed: 1) PMA was added directly to AM without transfer of supernatant, to prove that the loss of anti-NE activity of rSLPI was not just induced by PMA transferred with the PMN supernatant; and 2) rSLPI was incubated with resting AMs and activated PMN supernatant in the absence of AMs, respectively. The reaction time in these experiments was 32 min. As a comparison, rSLPI was incubated for 32 min with AMs that had been stimulated with PMA-activated PMN (for details see above).

Statistical analysis

For statistical comparison of the rSLPI and α_1-PI data, the Mann-Whitney U-test for independent samples was used. The results represent the arithmetic mean±SEM of two sets of experiments with all experiments performed in triplicate. A p-value of less than 0.05 was considered statistically significant. For experiments evaluating the impact of incubation of rSLPI with NCS and chloramine on the content in methionine sulphoxide residues, a multiple regression analysis was performed [25].

Results

N-chlorosuccinimide and chloramines

Exposure of rSLPI and α_1-PI to NCS (fig. 1) and chloramines (fig. 2) caused a loss of anti-NE activity. In both sets of experiments, the induced reduction in anti-NE activity was positively correlated to the molar ratio of oxidant to antiprotease. With NCS, rSLPI and α_1-PI gave similar results ($p>0.5$, all comparisons). With the highest concentrations of chloramines used, remaining anti-NE activity was higher for rSLPI than for α_1-PI without the differences reaching statistical significance ($p>0.6$, all comparisons).

Quantification of the methionine sulphoxide residues in the rSLPI samples treated with NCS or chloramines showed a significant (NCS: $p<0.002$; chloramines: $p<0.02$) inverse correlation between the anti-NE activity and the proportion of methionine sulphoxide residues as a percentage of total methionine (NCS: r=-0.98; fig. 3a; chloramines: r=-0.99; fig. 3b).

Activated polymorphonuclear leucocytes

Activated PMNs represent a potent source of reactive oxygen metabolites. We therefore incubated rSLPI

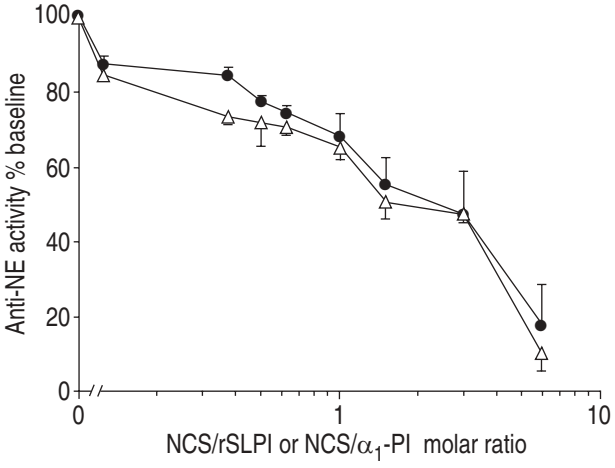

Fig. 1. – Inactivation of recombinant secretory leukoprotease inhibitor (rSLPI; ●) and α_1-protease inhibitor (α_1-PI; △) by N-chlorosuccinimide (NCS). Identical molar amounts of rSLPI and α_1-PI were included with varying amounts of NCS. The ratios of the molar concentration of NCS to the molar concentrations of rSLPI and α_1-PI and the corresponding antineutrophil elastase (NE) activity are shown. Data are presented as percentages of baseline value. Values are mean± SEM.

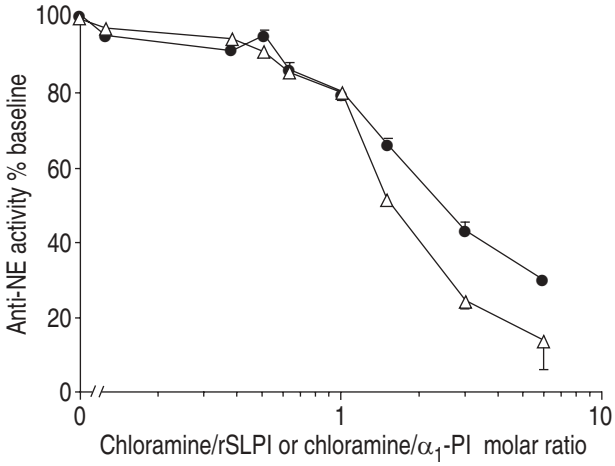

Fig. 2. – Inactivation of recombinant secretory leukoprotease inhibitor (rSLPI; ●) and α_1-protease inhibitor (α_1-PI;△) by chloramines. Identical molar amounts of rSLPI and α_1-PI were incubated with varying amounts of chloramines. The ratios of the molar concentration of chloramines to the molar concentrations of rSLPI and α_1-PI and the corresponding antineutrophil elastase (NE) activity are shown. Data are presented as percentages of baseline value. Values are mean±SEM.

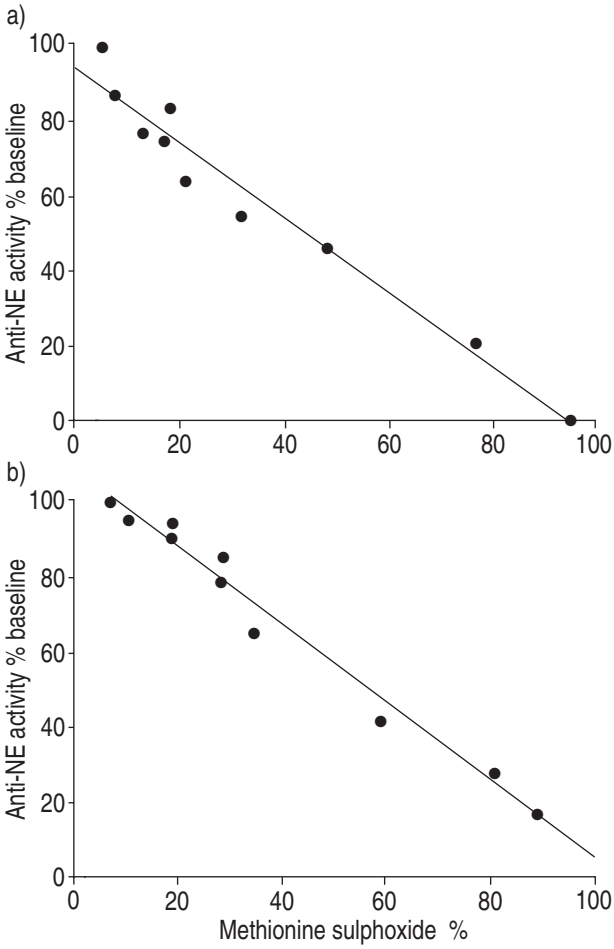

Fig. 3. – Quantification of methionine sulphoxide residues in samples containing recombinant secretory leukoprotease inhibitor (rSLPI) exposed to reactive oxygen metabolites. The number of methionine sulphoxide residues in the rSLPI samples treated with: a) N-chlorosuccinimide; or b) chloramines was evaluated as a percentage of the total methionine residues and plotted as a function of the corresponding antineutrophil elastase (NE) activity. Mean values and the calculated correlation lines are presented.

and α_1-PI with PMA-stimulated PMNs. With increasing reaction times between antiprotease and stimulated PMN, the remaining anti-NE activity of rSLPI tended to be higher than that of α_1-PI without the differences reaching statistical significance (for all comparisons p> 0.07; fig. 4). When a NE-specific substrate was added to the PMN supernatants, no significant NE activity could be recorded. Therefore, we conclude that these experiments were not influenced by NE released from PMN.

Activated alveolar macrophages

Incubation of rSLPI with primate AMs that had been activated by the supernatant of PMA-stimulated human PMNs caused a decline in anti-NE activity, which depended on the reaction time. There was a tendency of the resulting anti-NE activities to be smaller for α_1-PI than for rSLPI. Nevertheless, the differences did not reach statistical significance (for all comparisons p>0.9; fig. 5).

The loss of anti-NE activity observed in these experiments was not just induced by PMA transferred with the PMN supernatant. This was shown by adding PMA directly to AMs without transfer of supernatant. The resulting loss of activity of rSLPI was only about 25% of that seen in experiments with transfer of PMN supernatant (remaining anti-NE activity 85±3% *versus* 37±6%). When rSLPI was incubated with resting AMs (remaining anti-NE activity 83±10%) or PMN supernatant in the absence of AMs (remaining anti-NE activity 65± 10%), final anti-NE activity was about twice that in the experimental set-up with AMs and PMN supernatant (37±6%). In the transferred cell-free supernatant of PMA-stimulated human PMNs, 1.84±0.1 nmol of methionine sulphoxide (in a volume of 270 µL) could be generated by addition of pure methionine. In addition, a myeloperoxidase activity of $6.1\pm0.2\times10^{-3}$ U·mL^{-1} was found.

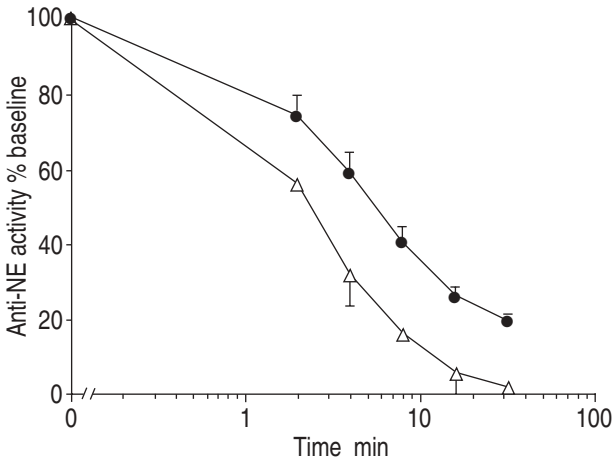

Fig. 4. – Inactivation of recombinant secretory leukoprotease inhibitor (rSLPI; ●) and α_1-protease inhibitor (α_1-PI; △) by activated polymorphonuclear cells (PMNs). Human peripheral blood PMNs were stimulated with phorbol myristate acetate (PMA) after identical molar amounts of rSLPI or α_1-PI had been added. Following varying incubation times, the reaction was stopped and supernatants were evaluated for their antineutrophil elastase (NE) activity. Data are presented as percentages of baseline value. Values are mean±SEM.

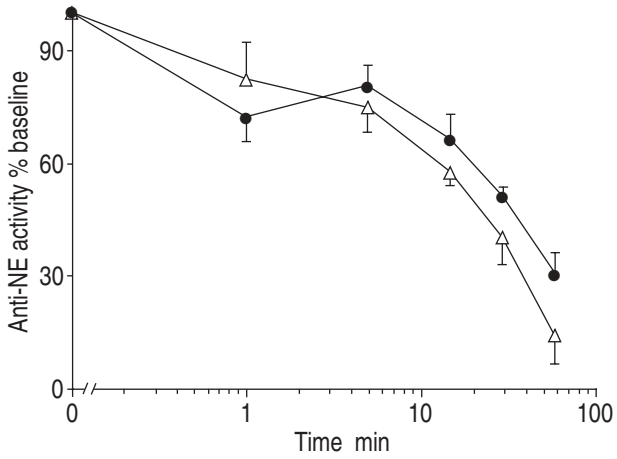

Fig. 5. – Inactivation of recombinant secretory leukoprotease inhibitor (rSLPI; ●) and α_1-protease inhibitor (α_1-PI; △) by activated alveolar macrophages (AMs). AMs obtained from primates by bronchoalveolar lavage were cultured, incubated with rSLPI and α_1-PI, and activated by cell-free supernatants of phorbol myristate acetate (PMA)-stimulated human polymorphonuclear cells (PMNs). Following varying incubation times, the reaction was stopped and supernatants were evaluated for their antineutrophil elastase (NE) activity. Data are presented as percentages of baseline value. Values are mean±SEM.

Discussion

In the present study, designed to directly compare the influence of reactive oxygen metabolites on the activity of rSLPI and α_1-PI, we found that both molecules demonstrated a similar loss of anti-NE activity under a variety of experimental conditions.

It is well known that α_1-PI may be partially inactivated by oxidation [3]. We have shown that about 67% of the SLPI molecules obtained from the epithelial surface of the normal human lung are inactivated, whereas, using similar methods, >95% of α_1-PI in BAL fluid is fully functional. Further experiments suggest that this partial inactivation of SLPI is caused by oxidation, leading to the conclusion that SLPI may be far more sensitive to reactive oxygen metabolites than α_1-PI [26]. KRAMPS *et al*. [27] incubated SLPI with stimulated PMNs, causing a reduction in anti-NE activity. In addition, the capacity of SLPI to form complexes with NE was diminished. These changes could be prevented by addition of catalase and methionine, but not superoxide dismutase, suggesting involvement of the myeloperoxidase system in this process. NADZIEJKO *et al*. [28] showed that following acute ozone exposure, anti-NE activity in BAL fluid provided by low-molecular-weight antiproteases including SLPI was reduced by 25%. Based on these observations it may be concluded that naturally occurring SLPI loses activity when exposed to oxidants. As rSLPI is identical to the natural form as regards structure and function [29], it should be as sensitive to these stress factors.

With this background, the goal of our study was to compare the loss of activity of rSLPI and α_1-PI under the influence of reactive oxygen metabolites. To thoroughly address this problem, we established several experimental models, using a chemical oxidant, a naturally occurring long-lived oxidant, activated PMNs and stimulated AMs. The latter experiments were designed

to model the influx of activated PMN into the lung. As shown, the loss of anti-protease activity induced by AMs incubated with PMN supernatant was more pronounced than in the experiments where AMs and PMN supernatant were incubated alone. This suggests that there is a relevant interaction between PMNs and AMs in this model. PMNs and AMs are a rich source of reactive oxygen species. They are capable of generating superoxide anion, produced by the membrane-bound enzyme nicotinamide adenine dinucleotide, reduced form (NADPH) oxidase, as well as nitric oxide [30]. Superoxide anion and other short-lived oxidants dismutated from superoxide anion (such as hydroxyl radicals) seem not to play a prominent role in the inactivation of proteinase inhibitors, as MAIER *et al*. [31] found that the xanthine oxidase/hypoxanthine/Fe(III) system has a very limited capability to inactivate α_1-PI. Nitric oxide does not behave as a strong oxidant toward most potential target molecules, either. However, when superoxide and nitric oxide combine, the potent long-lived oxidant peroxynitrite [32] is generated. As shown by MORENO and PRYOR [33], peroxynitrite is able to inactivate α_1-PI. These findings suggest that the synthesis of peroxynitrite is the major mechanism by which AMs may inactivate antiproteases. In contrast, WALLAERT *et al*. [34] demonstrated that PMA-stimulated AMs do not inactivate α_1-PI unless myeloperoxidase, released from PMNs, is present. In the cell-free PMN supernatant, we found myeloperoxidase activity and reactive oxygen metabolites, as indicated by the generation of methionine sulphoxide. This suggests that myeloperoxidase-dependent synthesis of long-acting oxidants occurred, although it should be noted that stimulated PMNs and AMs may release pathophysiologically important molecules other than oxidants, in particular a variety of proteases. In this context, besides NE, released matrix metalloproteinases such as gelatinase B and collagenase [35, 36] can cleave α_1-PI. Hence, the induced loss of activity could be at least partially caused by mechanisms other than oxidation. Nevertheless, we did not find any NE activity in our PMN supernatants, which supports reports by several authors that the release of NE caused by stimulation of PMN with PMA is minimal [37, 38].

In all applied test systems, the loss of anti-NE activity was similar for rSLPI and α_1-PI. This is not surprising, since the active centres of both SLPI (Met^{73})[14] and α_1-PI (Met^{358})[1] carry methionine residues. It has clearly been demonstrated by other authors that with α_1-PI, oxidation of this methionine residue induces a dramatic loss of activity [3, 39]. By quantifying the methionine sulphoxide residues we showed that oxidation of rSLPI occurs under the experimental conditions used. The observed inverse correlation between anti-NE activity and methionine sulphoxide content supports data from other groups: GONIAS *et al*. [40] evaluated the effects of incubation of SLPI with oxidants selective for methionine residues, *e.g*. cisplatinum (II) diaminechloride; and KRAMPS *et al*. [41] demonstrated a dose-dependent loss of antiprotease function. In another study, mutants of rSLPI were tested in which the methionine residue in the active centre was replaced by the nonoxidizable amino acid leucin. Incubating this mutant with cisplatinum (II) diaminechloride as well as triggered PMNs, resulted in reduced loss of activity compared with unmodified rSLPI. These results were confirmed in *in vivo* experiments: in animal emphysema models with intratracheal instillation of NE and lipopolysaccharide, the protective effect of the oxidant-resistant rSLPI mutant was superior to that of unmodified rSLPI [42, 43]. These data suggest that methionine residues are critical for the anti-NE activity of both α_1-PI and rSLPI.

The findings of the present study indicate that the loss of activity in the milieu of the diseased lung cannot be considered as a factor in making the decision to use either recombinant secretory leukoprotease inhibitor or α_1-protease inhibitor for therapeutic purposes. Nevertheless, in disease states with a high load of oxidants, recombinant secretory leukoprotease inhibitor may prove to be superior to α_1-protease inhibitor. There are data suggesting that recombinant secretory leukoprotease inhibitor is capable of behaving not only as an antiprotease, but also as an antioxidant. This is probably secondary to its high content of cysteine residues, that may be set free during the degradation of the recombinant secretory leukoprotease inhibitor molecule, which in the sheep model serves as a slow-release form of glutathione, thereby augmenting the pulmonary antioxidant protective screen provided by the glutathione system [44]. However, the relevance of this finding in human disease is not yet known.

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