Mutants of Plasminogen Activator Inhibitor-1 Designed to Inhibit Neutrophil Elastase and Cathepsin G Are More Effective in Vivo than Their Endogenous Inhibitors*

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Neutrophil elastase and cathepsin G are abundant intracellular neutrophil proteinases that have an important role in destroying ingested particles. However, when neutrophils degranulate, these proteinases are released and can cause irreparable damage by degrading host connective tissue proteins. Despite abundant endogenous inhibitors, these proteinases are protected from inhibition because of their ability to bind to anionic surfaces. Plasminogen activator inhibitor type-1 (PAI-1), which is not an inhibitor of these proteinases, possesses properties that could make it an effective inhibitor of neutrophil proteinases if its specificity could be redirected. PAI-1 efficiently inhibits surface-sequenced proteinases, and it efficiently mediates rapid cellular clearance of PAI-1-proteinase complexes. Therefore, we examined whether PAI-1 could be engineered to inhibit and clear neutrophil elastase and cathepsin G. By introducing specific mutations in the reactive center loop of wild-type PAI-1, we generated PAI-1 mutants that are effective inhibitors of both proteinases. Kinetic analysis shows that the inhibition of neutrophil proteinases by these PAI-1 mutants is not affected by the sequestration of neutrophil elastase and cathepsin G onto surfaces. In addition, complexes of these proteinases and PAI-1 mutants are endocytosed and degraded by lung epithelial cells more efficiently than either the neutrophil proteinases alone or in complex with their physiological inhibitors, α1-proteinase inhibitor and α1-antichymotrypsin. Finally, the PAI-1 mutants were more effective in reducing the neutrophil elastase and cathepsin G activities in an in vivo model of lung inflammation than were their physiological inhibitors.

Neutrophils are the first defensive cells to extravasate from the circulation into infected areas where their primary role is to ingest foreign particles and eliminate them by using an arsenal of bactericidal, hydrolytic, and oxidative agents (1–3). The direct action of neutrophils is temporary because they degranulate soon after reaching the affected area, but their intracellular proteinases can have a lasting effect.

In many chronic inflammatory disorders where there is a persistent influx and degranulation of neutrophils, their proteinases can overwhelm endogenous proteinase inhibitors, cause tissue degradation, and augment the inflammatory response. The broad substrate specificities of neutrophil elastase and cathepsin G are similar to the digestive proteinases, pancreatic elastase and chymotrypsin, respectively. Like their pancreatic counterparts, the neutrophil proteinases can degrade most proteins, including cross-linked extracellular matrix proteins such as collagens and elastin. In addition to degrading extracellular matrix proteins, these neutrophil proteinases can also intensify the host inflammatory response by both proteolytic and non-proteolytic mechanisms (4–7).

The activities of neutrophil elastase and cathepsin G are primarily regulated by the serine proteinase inhibitors (serpins), α1-proteinase inhibitor (α1 PI, also called α1antitrypsin) and α1-antichymotrypsin (α1 ACT), respectively. These inhibitors are abundant plasma proteins, present in the circulation at high concentrations (8). The importance of these inhibitors in regulating neutrophil proteinase activity in extracellular tissues has been demonstrated in individuals with α1 PI deficiency who are more likely to develop early onset emphysema because of degradation of lung elastin (9, 10).

Neutrophil elastase and cathepsin G are very basic proteins (pI ≥ 8.5) that bind negatively charged molecules like DNA and heparin with high affinity. Their binding to these anionic surfaces limits the accessibility of their active site to both small substrates and serpins. α1 PI and α1 ACT are very effective inhibitors of neutrophil elastase and cathepsin G in solution phase, but efficiency is greatly reduced in the presence of these surfaces. The reason for this is thought to be because of the phase separation of neutrophil elastase and cathepsin G from α1 PI and α1 ACT due to the inability of the inhibitors to bind these surfaces. Partitioning of these proteinases from their endogenous inhibitors could explain the tissue damage associated with many chronic inflammatory disorders because they are protected from inhibitors by anionic macromolecules present in cell debris (11–13).

Unlike α1 PI and α1 ACT, which are efficient inhibitors of proteinases in solution phase, other serpins are efficient inhibi-

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The abbreviations used are: α1 PI, α1-proteinase inhibitor; PAI-1, plasminogen activator inhibitor type-1; α1 ACT, α1-antichymotrypsin; LRP, low-density lipoprotein receptor-related protein; PAI-1Aβ, PAI-1V343A A346V; PAI-1P, PAI-1R346F; PAI-1A, PAI-1R346A; FMLP, formyl-Met-Leu-Phe; RAP, receptor-related protein; PMSF, phenylmethylsulfonyl-fluoride; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; LDL, low density lipoprotein; NE, neutrophil elastase; serpin; RCL, reactive center loop.
itors of their target proteinases when they are bound to a surface. A classic example is antithrombin-III, whose rate of inhibition is enhanced by heparin (14). Another serpin that is able to inhibit proteinases bound to surfaces is plasminogen activator inhibitor type-1 (PAI-1), which is the principal inhibitor of the plasminogen activators (PAs), urokinase-type plasminogen activator and tissue-type plasminogen activator in vivo (15, 16). Both PAs bind to a variety of surfaces, including cellular receptors, fibrin, and heparin. Surface binding, however, does not protect them from inhibition by PAI-1 (17–20). Besides the ability of PAI-1 to inhibit PAs bound to receptors and surfaces, PAI-1 is able to mediate rapid cellular clearance of the target proteinase.

Upon complex formation with a proteinase, PAI-1 undergoes a rapid conformational change that increases its affinity for the clearance receptors of the low density lipoprotein (LDL) receptor family. These include the LDL receptor-related protein (LRP), the very low density lipoprotein receptor, and Megalin (21–23). We previously showed that the high affinity binding of PAI-1-proteinase complexes to LRP was independent of the proteinase but was mediated through a cryptic site on PAI-1 that is exposed when it is in a covalent complex with a proteinase. Furthermore, this enhanced clearance was significantly more efficient compared with clearance of proteinases, such as thrombin, in complex with other serpins, including antithrombin-III, α1-PI, and heparin cofactor-II (22). Thus, PAI-1 not only inhibits proteinases but also specifically promotes their clearance.

In this study we have taken advantage of these innate properties of PAI-1, which normally is cleaved by these proteinases, and designed mutations in the reactive center loop (RCL) of PAI-1, which converts it to an efficient inhibitor of either pancreatic elastase or chymotrypsin or of the neutrophil proteinases, neutrophil elastase and cathepsin G. The efficiency of these PAI-1 mutants is not adversely affected by surfaces such as heparin or DNA, and they are markedly more efficient at promoting the cellular clearance and degradation of neutrophil proteinases compared with their natural serpin inhibitors. Together, these properties make the PAI-1 mutants more effective in vivo than their endogenous inhibitors at reducing the neutrophil proteinase concentrations in a model of lung inflammation.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Active human neutrophil elastase, cathepsin G, and α1-antichymotrypsin were purchased from Athens Biochemicals (Athens, GA). Chromogenic substrates N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (for cathepsin G and chymotrypsin), N-succinyl-Ala-Ala-Pro-Val-p-nitroanilide (for neutrophil elastase), N-succinyl-Ala-Ala-Ala-N-p-nitroanilide (for pancreatic elastase), bovine serum albumin, bovine chymotrypsin, hexadecyl-trimethyl ammonium bromide, endo-nitroanilide (for pancreatic elastase), bovine serum albumin, and bovine β-casein were generous gifts from Dr. D. K. Strickland (J. H. Holland Laboratory). These PAI-1 mutants were expressed and purified as described (24) and isolated and purified as described by Kivistö and Shore (25). Preparations with significant endotoxin levels as determined by coagulase kit (Bio-Whittaker, Walkersville, MD) following the phenyl-Sepharose step were further purified by either additional passes through phenyl-Sepharose or Detoxi-Gel (Pierce) until endotoxin levels were at or below 10 units/ml.

**Biacore Analysis of the Binding of the Proteinases, Inhibitors, and Their Complexes to LRP**—Affinities of pancreatic elastase, neutrophil elastase, chymotrypsin, and cathepsin G for LRP either pancreatic elastase or chymotrypsin or of the neutrophil elastase (RCL) of PAI-1, which converts it to an efficient inhibitor of α1PI, α1ACT, PMSF, or PAI-1 mutants were measured by surface plasmon resonance using a BIA 3000 optical biosensor (Biacore AB, Uppsala, Sweden). Purified human LRP was immobilized at the level of 1000 response units. Remaining binding sites were blocked by 1 M ethanolamine, pH 8.5, and unbound proteinases were washed out with 0.5% SDS. Flow cells with immobilized ovalbumin at the level of 500 response units was used as a control for nonspecific protein binding. All binding reactions were performed in standard HBS-P buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, and 0.005% Tween 20. Binding of proteinases, inhibitors, and their complexes to LRP was measured at 25°C at a flow rate of 30 μl/min for 4 min, followed by 4 min of dissociation. Chip surfaces were regenerated with subsequent 1-min pulses of 1 M NaCl, pH 4.0, and 1 M NaCl containing 10 mM NaOH, followed by 2 min of washing with HBS-P. Binding of proteinases, inhibitors, and proteinase-inhibitor complexes was measured using a range of concentrations (10–50 μM). Collected data were analyzed with BIA evaluation 3.0 software (Biacore) using global analysis to fit a 1:1 Langmuir binding model with mass transfer limitation.

**Cellular Endocytosis and Degradation Assays of Proteinases**—A rat pretype-II pneumocyte cell line (T-II) was a generous gift from Dr. R. K. Mallampalli (University of Iowa College of Medicine, Iowa City, IA) (26). These cells were grown in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) containing penicillin and streptomycin. For endocytosis and degradation studies, T-II cells were seeded onto 12-well plates, (1–3 × 10⁶ cells/well) coated with 0.1% gelatin and allowed to adhere for 18 h at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin. Before addition of radiolabeled proteinases, cells were washed twice with serum-free Dulbecco’s modifed Eagle’s medium and incubated for 30 min in serum-free Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin before addition of either preformed ¹²⁵I-labeled proteinase-inhibitor complexes or ¹²⁵I-labeled proteinases. Where indicated, RAP (1 μg/ml) was added prior to the ¹²⁵I proteinase-inhibitor complexes.

Quantitation of the endocytosed and degraded ligand was done as described (27) with minor modifications. Briefly, cellular degradation of the ¹²⁵I-labeled proteinases was determined by removing the medium and precipitating it using 10% trichloroacetic acid and 5% phosphotungstic acid (final concentration). Acid-soluble radioactivity was taken to represent the amount of endocytosed ligand. To evaluate the effects of α1PI and PAI-1-W on the endocytosis and degradation of exogenously added ¹²⁵I elastase, cells were grown in medium containing 10% serum as described above. Washed monolayers were then incubated with ¹²⁵I elastase (10 nM) for 30 min, after which increasing concentrations of either α1PI or PAI-1-W were added and incubated for 4 h at 37°C to determine endocytosis or 18 h for degradation.

Kinetics of Proteinase Inhibition by α1PI, α1ACT, and PAI-1 Mutants—Before measuring the second order rate constant for the inhibition of the neutrophil proteinases, the stoichiometry of the inhibitor as a proteinase substrate to a proteinase inhibitor (SI value) was determined. Briefly, a constant concentration of the neutrophil proteinases was incubated with an increasing concentration of the proteinase inhibitors in the presence or absence of heparin or DNA. After 30 min the appropriate chromogenic proteinase substrate was added to the reaction mixture, and the proteinase activity was measured. The results were plotted as activity of the proteinase versus the inhibitor concentration divided by the enzyme concentration. The intercept on the x-axis indicates an indicator of whether the inhibitor is competitive or noncompetitive or whether higher concentrations of the inhibitors are required.

Two methods were used to obtain kinetic parameters for the inhibition of the proteinases. For slow reactions (k_s < 10⁻⁷), the PAI-1 mutants will be assumed to inhibit target proteinases by the two-step inhibitory
mechanism first described for irreversible inhibitors by Kitz and Wilson
(28) and Hastings et al. (29).

For fast reactions between the proteins and the inhibitors (ki > 107), the scheme described in Lawrence et al. (24) was used. The effects of heparin and DNA on the kinetics of inhibition were determined in the presence of either 0.1 mg/ml heparin or 0.1 mg/ml DNA.

In Vivo Lung Inflammation Assay—In vivo lung inflammation assays were performed essentially as described (31), with some modifications. Briefly, male C57BL/6J mice (age 8–12 weeks) under anesthesia were intranasally instilled with either PBS or a mixture of endotoxin and fMLF, 100 and 50 µg, respectively, in a total volume of 150 µl in PBS. PAI-1AV and PAI-1F mutants, 50 µg/25 µg, respectively, or α,AP/α,ACT, 100 µg/25 µg, respectively, in a total volume of 150 µl in PBS were administered along with the endotoxin/fMLF instillation mixture. Additionally, PAI-1AV/PAI-1F, 100 µg/25 µg, respectively, or α,AP/α,ACT, 100 µg/25 µg, respectively, in a total volume of 150 µl or PBS were injected intraperitoneally to both PBS- and endotoxin/fMLF-treated mice immediately following nasal instillation and again 16–17 h postinstillation. At 24–26 h postinstillation, animals were anesthetized and perfused with PBS. Lungs were then excised, washed briefly and the rate of inhibition of PAI-1A in vivo was determined by subtracting the activities of animals treated intranasally with PBS from the endotoxin/fMLF-treated animals, and with or without added proteinase inhibitors. (Approved Animal Welfare Assurance A3379–01 registered with the Public Health Services, Office of Laboratory Animal Welfare).

RESULTS AND DISCUSSION

Generation and Characterization of PAI-1 Mutants That Inhibit Pancreatic and Neutrophil Proteinases—Serpins act as “suicide inhibitors” because when a proteinase cleaves a serpin at a site within the RCL, termed the scissile bond (P1-P1’), it traps the proteinase in a non-reversible covalent complex (32, 33). In many cases the amino acid composition of the serpin scissile bond reflects the substrate specificity of the target proteinase. The scissile bond of wild-type PAI-1 is Arg-346-Met-347, which enables it to inhibit many serine proteinases with trypsin-like specificities, but kinetic analysis showed that it is the most effective inhibitor of both PAs (34). Because PAI-1 is an efficient inhibitor of its target proteinases both in solution and in solid phase (17–20) and because it is very efficient at promoting their cellular clearance, (27), we wanted to determine whether we could redirect PAI-1 specificity toward neutrophil elastase and cathepsin G, which wt PAI-1 does not inhibit, (35, 36), and still retain these properties.

Mutations in a serpin scissile bond have been shown to redirect it to target another proteinase (37). Therefore we tested whether a PAI-1 mutant with the P1 Arg-346 residue changed to an alanine (PAI-1R346A, referred herein as PAI-1Av) (38), could inhibit pancreatic elastase, which prefers Ala as the P1 residue. The PAI-1Av has no inhibitory activity against urokinase-type plasminogen activator (data not shown) but retained the ability to bind vitronectin with affinity, equal to wild-type PAI-1 (39). The rate of inhibition (ki) of pancreatic elastase by PAI-1Av is 4.6 × 108 M⁻¹ s⁻¹, which is comparable with the rate obtained by Laurent and Bieth (40) for α,PI (4.7 × 108 M⁻¹ s⁻¹). These results demonstrate that the specificity of PAI-1 can be redirected toward pancreatic elastase with a rate of inhibition similar to α,PI. To target serine proteinases with chymotrypsin-like specificities, a second PAI-1 mutant was generated with a phenylalanine at the P1 position (PAI-1R346F, referred herein as PAI-1F). This mutant was tested against chymotrypsin, and the rate of inhibition of PAI-1F was 6.1 × 108 M⁻¹ s⁻¹ compared with 8.1 × 108 M⁻¹ s⁻¹ for α,ACT reported by Rubin et al. (41).

The results reported above demonstrate that the proteinase specificity of PAI-1 can be easily redirected by changing the amino acid composition of the P1 residue without a substantial loss in the rate of inhibition in solution phase. Therefore, these PAI-1 mutants were tested for their ability to inhibit other serine proteinases with similar specificity, specifically neutrophil elastase and cathepsin G. Kinetic analysis of PAI-1Av inhibition of neutrophil elastase yielded a ki of 1.4 × 108 M⁻¹ s⁻¹, which is about 300-fold less than the rate of inhibition of pancreatic elastase by PAI-1Av (ki = 4.6 × 108 M⁻¹ s⁻¹) and is ~10,000 fold less than α,PI inhibition of neutrophil elastase (ki = 1.3 × 107 M⁻¹ s⁻¹). The decrease in the ability of PAI-1Av to inhibit neutrophil elastase was surprising because it efficiently inhibited pancreatic elastase at a rate equal to that of α,PI. This discrepancy could be because of the preference of neutrophil elastase for valine over alanine as the P1 residue or because the proteinase was cleaving the RCL at another site than the P1-P1’, rendering it inactive as was demonstrated for wt-PAI-1 (35). Sequence analysis of the reaction mixture of PAI-1Av with neutrophil elastase showed that PAI-1Av was being cleaved at both the Val-343-Ser-344 position (P3-P4) and at Ala-346-Met-347 (P1-P1), indicating that the inefficiency of PAI-1Av might be due in part to this non-productive cleavage at Val-343-Ser-344, coupled with the P1 alanine being a suboptimal residue for neutrophil elastase.

To improve PAI-1Av as an inhibitor of neutrophil elastase, the Val-343 residue was replaced with alanine and the Ala-346 was replaced with valine. The resulting mutant, PAI-1V343A/Val, has the non-productive Val-343 cleavage site changed to alanine, which is a suboptimal cleavage site for neutrophil elastase, and the alanine at the P1 position changed to valine, which is preferred by neutrophil elastase as demonstrated by small peptide substrates (43). Valine was also chosen as the P1 residue because studies by Shubeita et al. (44) demonstrated that replacing the P1-P1’ residues of wt-PAI-1 with those of α,PI (Met-Ser) did not convert PAI-1 into an inhibitor of elastase or trypsin. Additionally, the PAI-1Av mutant does not alter the length of the PAI-1 RCL, which is critical for its ability to inhibit its target proteinases (45).

The kinetic analysis of the PAI-1Av inhibition of neutrophil elastase showed an approximate 400-fold improvement in the
Mutant PAI-1 Inhibitors of Neutrophil Proteinases

Table II

| Complexes | $k_{on}$ | $k_{off}$ | $K_A$ | $K_D$ |
|-----------|---------|---------|-------|-------|
| NE:PAI-1AV | $5.4 \times 10^7$ | $8.1 \times 10^{-3}$ | $6.7 \times 10^9$ | $1.5 \times 10^{-10}$ |
| CG:PAI-1 | $2.5 \times 10^6$ | $3.3 \times 10^{-3}$ | $7.6 \times 10^8$ | $1.3 \times 10^{-9}$ |
| CT:PAI-1 | $1.0 \times 10^6$ | $5.8 \times 10^{-4}$ | $1.7 \times 10^9$ | $5.8 \times 10^{-10}$ |
| PE:PAI-1 | $9.5 \times 10^5$ | $1.2 \times 10^{-3}$ | $7.9 \times 10^8$ | $1.3 \times 10^{-9}$ |
| NE:αPI | >$10^4$ | | | |
| NE:αACT | >$10^4$ | | | |
| CG:αPI | >$10^4$ | | | |
| CG:αACT | >$10^4$ | | | |
| CT:αPI | >$10^4$ | | | |
| CT:αACT | >$10^4$ | | | |
| PE:αPI | >$10^4$ | | | |
| PE:αACT | >$10^4$ | | | |

and α1ACT are superior inhibitors of the neutrophil proteinases in solution, the presence of anionic surfaces, which are likely present at sites of inflammation in vivo, greatly effect α1PI and α1ACT inhibition but do not severely affect the PAI-1AV and PAI-1F mutants.

Binding of Neutrophil Elastase and Cathepsin G and their Complexes to LRP and Their Clearance by Lung Epithelial Cells in Vitro—Previously we demonstrated that when PAI-1 is in a covalent complex with a proteinase, it binds LRP with high affinity and is cleared by cells more effectively than proteinases in complex with other serpins or with synthetic inhibitors. This clearance is thought to be mediated by a cryptic high affinity binding site for LRP that is exposed in PAI-1 only when it is in a covalent complex with a proteinase (27). To determine whether PAI-1AV and PAI-1F retained the ability of wtPAI-1 to promote cellular clearance when in complex with these proteinases, we measured their affinities for LRP and their cellular endocytosis and degradation compared with α1PI and α1ACT.

Before analyzing the affinities of the serpin-proteinase complexes, the equilibrium dissociation constants for each individual serpin and proteinase were measured by their binding to immobilized LRP as measured by surface plasmon resonance. The results demonstrate that the PAI-1 mutants show weak binding to LRP ($K_D \sim 0.2 \mu M$) and that the binding of α1PI and α1ACT was also weak, showing $K_D$s greater than $10^{-4} \mu M$. Of the proteinases, neutrophil elastase showed moderate binding affinity to LRP ($K_D \sim 0.065 \mu M$) with pancreatic elastase, chymotrypsin, and cathepsin G showing a $K_D > 10^{-4} \mu M$.

Fig. 1 shows representative surface plasmon resonance binding curves of neutrophil elastase in complex with either PAI-1AV or α1PI (Fig. 1, A and B, respectively) binding to immobilized LRP. These data show that neutrophil elastase in complex with α1PI bound weakly to the LRP sensor chip, whereas neutrophil elastase in complex PAI-1AV showed high affinity binding. The binding affinities of the proteinase:serpin pairs are summarized in Table II. These data indicate that both pancreatic and neutrophil proteinases in complex with the PAI mutants bind LRP with nanomolar to picomolar affinities, whereas proteinases in complex with either α1PI or α1ACT exhibited only very weak binding to LRP.

Because previous studies have shown that high affinity binding of PAI-1 in complex with a proteinase to immobilized LDLRs in vitro can predict efficient cellular clearance, we wanted to determine whether these PAI-1 mutants also promote the cellular clearance of their proteinase-inhibitor complexes. For these studies the pancreatic and neutrophil proteinases were radiolabeled and preincubated with either α1PI, α1ACT, PAI-1AV, PAI-1F, or PMSF. These premade complexes were then added to a lung epithelial cell line generated from rat pre-
type-II pneumocytes (26); the degradation of the proteinase was measured in the presence or absence of RAP, an antagonist of ligand binding to LDLR (48). The cell-mediated degradation of each proteinase in complex with inhibitor indicated that all PAI-1-proteinase complexes are degraded by the pneumocytes ~10–30-fold more efficiently than proteinases in complex with α₁PI, α₁ACT, or PMSF (Fig. 2, A–D, respectively). Additionally, the cellular degradation was mediated through internalization by an LDLR family member because the inclusion of RAP blocked the degradation of the PAI-I mutant proteinase com-

Fig. 2. Cellular degradation of proteinase-inhibitor complexes by type-II pneumocytes. ¹²⁵I-labeled pancreatic elastase (PE, panel A), chymotrypsin (CT, panel B), neutrophil elastase (NE, panel C), or cathepsin G (GC, panel D) was incubated with either a 2-fold molar excess of corresponding serpin inhibitors or 1 μM PMSF for 20 min at 25 °C before addition to cells. Where indicated, RAP (1 μM) was added 30 min prior to addition of either ¹²⁵I-labeled proteinases or their complexes, and the cells were incubated for 18–22 h at 37 °C, 5% CO₂ after which cellular degradation of proteinases was measured as described under “Experimental Procedures.”

Fig. 3. Comparison of cellular endocytosis and degradation of neutrophil elastase mediated by α₁PI and PAI-1AV. Active ¹²⁵I-labeled neutrophil elastase or PMSF-treated ¹²⁵I-labeled neutrophil elastase (100 nM) was added to a monolayer of type-II pneumocytes and incubated for 30 min, after which either α₁PI (○) or PAI-1AV (●) was added at the indicated concentrations. Endocytosis was determined after 2.5 h of incubation (A) and degradation after 22 h of incubation (B). Values obtained from neutrophil elastase treated with PMSF were subtracted from those obtained with neutrophil elastase in complex with α₁PI and PAI-1AV.
plexes. Because the above study was performed using pre- 
formed complexes of radiolabeled proteinase with inhibitors, we 
wanted to determine the efficiency of the PAI-1 mutants to 
inhibit and mediate the cellular clearance of a free proteinase.

For these studies $^{125}$I-labeled neutrophil elastase was added 
to a cell layer of the type-II pneumocytes followed by the addi-
tion of increasing concentrations of either $\alpha_{1}$PI or PAI-1$^{AV}$. As 
is seen in Fig. 3, A and B, increased concentrations of the 
PAI-1$^{AV}$ mutant added to the cells showed a concentration-de-
pendent increase in the endocytosis and degradation of the 
labeled neutrophil elastase. In contrast, increasing concen-
trations of $\alpha_{1}$PI did not result in an enhancement of neutrophil 
elastase cellular clearance.

The results shown in Figs. 2 and 3 demonstrate that cellular 
clearance of neutrophil proteinases in complex with PAI-1 mu-
tants can be mediated by lung epithelial cells because they 
express members of the LDLR family (49–51). This indicates 
that the clearance of PAI-1-proteinase-inhibitor complexes can 
occur locally rather than through hepatic receptors as is the 
case for most plasma proteinase-inhibitor complexes (52).

PAI-1$^{AV}$ and PAI-1$^{F}$ Reduce the Neutrophil Proteinase Ac-
itivities in an in vivo Model of Acute Lung Inflammation—The in 
vitro studies presented above demonstrate that PAI-1$^{AV}$ and 
PAI-1$^{F}$, in the presence of anionic polymers such as proteogly-
cans and DNA, are efficient inhibitors of neutrophil elastase 
and cathepsin G. In addition, these PAI-1 mutants were much 
more efficient at mediating the cellular clearance of the 
neutrophil proteinases than $\alpha_{1}$PI or $\alpha_{1}$ACT. Therefore, to see 
whether their efficiency in vitro could also be demonstrated in 
vivo, we examined their ability to reduce neutrophil proteinase 
activities in a mouse model of acute lung inflammation through 
intranasally instilled lipopolysaccharide (LPS) and a fMLF 
peptide.

Instillation of LPS and/or fMLF into lungs has been previ-
ously used as a model to study the effects inflammatory 
cells have on the structure and function of lungs. Both LPS and 
fMLF are bacterial products that are recognized by many cells 
as signs of infection, to which many cells respond by secreting 
chemokines, which attract neutrophils and other defensive 
cells (53, 54). In addition to responding to agents secreted 
by cells in response to bacterial products, inflammatory cells 
express receptors that recognize these bacterial products, which 
stimulates their extravasation from the circulation toward 
the focus of the infection.

For these studies, mice were intranasally instilled with LPS 
and fMLF in combination with either $\alpha_{1}$PI and $\alpha_{1}$ACT or PAI-
1$^{AV}$ and PAI-1$^{F}$, in conjunction with intraperitoneal injections 
of these inhibitor pairs. The mice received a second intraperi-
toneal injection of proteinase inhibitors 16–17 h later, and the 
neutrophil proteinase levels in the lungs were determined 
24–26 h after the initial instillations.

Fig. 4 shows the effects of human $\alpha_{1}$PI, $\alpha_{1}$ACT and PAI-1$^{AV}$/ 
PAI-1$^{F}$ on the neutrophil elastase and cathepsin G activities in 
the lungs of mice instilled intranasally with endotoxin and 
fMLF peptide. PAI-1$^{AV}$/PAI-1$^{F}$ significantly decreased protein-
ase activities ($p < 0.05$), whereas there was no significant 
decrease of proteinase activity in lungs treated with $\alpha_{1}$PI/ 
$\alpha_{1}$ACT compared with controls ($p > 0.1$). These data demon-
strate that the PAI-1 mutants are more efficient at reducing 
the load of neutrophil proteinases in the lungs in vivo than 
 injections of equivalent amounts of $\alpha_{1}$PI and $\alpha_{1}$ACT. This 
is notable because the mice used in this study have a full com-
plement of endogenous plasma neutrophil proteinase inhibitors 
(42) and enhancing their levels to those of their human 
counterparts does not influence the outcome. However, injecting 
the mice with the engineered PAI-1 variants significantly reduced 
the proteinase burden in the lungs. Thus, in this study we show 
that PAI-1 can be engineered to be an effective inhibitor of 
neutrophil proteinases in environments where the endogenous 
inhibitors, $\alpha_{1}$PI and $\alpha_{1}$ACT, are impaired. Additionally, our 
results demonstrate that redirecting the specificity of PAI-1, 
which has distinct capabilities such as the ability to inhibit 
surface-bound proteinases and mediate their efficient cellular 
clearance, can yield potentially superior inhibitors of protein-
ases involved in pathologies.

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Mutants of Plasminogen Activator Inhibitor-1 Designed to Inhibit Neutrophil Elastase and Cathepsin G Are More Effective \textit{in Vivo} than Their Endogenous Inhibitors

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