Oxidative Regulation of Neutrophil Elastase–Alpha-1-Proteinase Inhibitor Interactions

Peter J. Ossanna, Samuel T. Test, Nancy R. Matheson,* Sandra Regiani, and Stephen J. Weiss
*Division of Hematology/Oncology, Department of Internal Medicine, Simpson Memorial Research Institute, University of Michigan, Ann Arbor, Michigan 48109; *Department of Biochemistry, University of Georgia, Athens, Georgia 30602*

Abstract

Triggered human neutrophils were able to maintain released elastase in an active form in the presence of purified alpha-1-proteinase inhibitor (α-1-PI), serum or bronchoalveolar lavage fluid (BAL). The accumulation of free elastase activity was associated with a decrease in the ability of the α-1-PI to inhibit porcine pancreatic elastase, an increase in proteinase activity associated with α-2-macroglobulin, and the oxidation of α-1-PI to a molecule containing four methionine sulfoxide residues. Neutrophils used both hypochlorous acid and long-lived *N*-chloroamines to oxidize the α-1-PI, but hypochlorous acid was preferentially used for suppressing the activity of the antiproteinase over short distances whereas the *N*-chloroamines were effective even when the phagocytes and α-1-PI were physically separated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified α-1-PI, serum, or BAL that had been incubated with triggered neutrophils revealed that the released neutrophil elastase was not complexed with the antiproteinase and that a portion of the α-1-PI had undergone proteolysis. These data suggest that the presence of free neutrophil elastase as well as inactive, oxidized,and proteolyzed α-1-PI in fluids recovered from inflammatory sites in vivo could be directly mediated by triggered neutrophils alone.

Introduction

Neutrophil elastase can exert powerful proinflammatory effects by virtue of its ability to degrade connective tissue components, attack serum proteins, and alter cellular functions (1–3). In vivo, neutrophil elastase is normally regulated by the plasma antiproteinase, alpha-1-proteinase inhibitor (α-1-PI),[1] which rapidly complexes and inactivates the free enzyme (4). However, in a growing number of inflammatory disease states, recovered tissue fluids have been shown to contain free neutrophil elastase activity (for recent examples see References 5–16). In association with these findings, analyses of the α-1-PI status in these pathologic samples revealed the presence of uncomplexed, inactive, and proteolyzed antiproteinase (5–16). Because the loss of α-1-PI function represents a pivotal step in the pathogenesis of elastase-mediated damage, increasing attention has focused on the identification of those processes capable of inactivating the antiproteinase. Recent studies have underlined the potential role of two leukocyte populations in the down-regulation of α-1-PI activity at inflammatory sites: the macrophage and the neutrophil. Macrophages may use either cathepsin B or the metalloproteinase, elastase, to fragment the antiproteinase to an inactive form (17–19). In this scenario, macrophage-mediated α-1-PI inactivation would allow released neutrophil elastase to degrade host tissues in an uncontrolled manner. Alternatively, neutrophils themselves can directly suppress α-1-PI activity by generating reactive oxygen metabolites that are capable of oxidizing a critical methionine residue in the antiproteinase (20–24). However, although oxidized α-1-PI is usually considered to be completely inactive, the modified antiproteinase retains considerable neutrophil elastase inhibitory capacity (4, 25). Based on the known rate constants of association between purified neutrophil elastase and oxidized α-1-PI, Beatty et al. have calculated that the oxidation of physiologic concentrations of the antiproteinase increases the half-life of elastase from 0.64 ms to 1.3 s (4, 25). These kinetic analyses demonstrate that the oxidation of α-1-PI would allow released neutrophil elastase to bind rapidly to any adjacent connective tissue components for which it has a strong affinity. But, if released elastase remains free in solution, the active proteinase would not be expected to accumulate in the presence of an excess of oxidized α-1-PI. Thus, the ability of the neutrophil to directly modify α-1-PI in a manner that would explain the presence of free elastase activity in fluids obtained from a variety of inflamed sites is unclear. In this study, we have examined each of the interactions among neutrophil-derived oxidants, neutrophil elastase, and native or oxidized α-1-PI in an intact cell system. Our results demonstrate that (*a*) neutrophils triggered in the presence of an excess of either purified α-1-PI, serum, or bronchoalveolar lavage (BAL) fluid can directly accumulate and maintain released elastase in an active form and (*b*) oxidation and proteolysis of the native antiproteinase play an important role in these events.

Methods

Special materials

Alpha-1-PI was purified from outdated human plasma as previously described (26) or obtained commercially (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). Both preparations of α-1-PI migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), exhibited equal porcine pancreatic elastase (PPE) inhibitory capacity, and yielded identical amino acid profiles after

Address reprint requests to Dr. Weiss, Simpson Memorial Institute, 102 Observatory Lane, Ann Arbor, MI 48109.

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1. *Abbreviations used in this paper:* AAPVCK, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-chloromethylketone; α-1-PI, alpha-1-proteinase inhibitor; α-1-PI$_f$, alpha-1-proteinase inhibitor fragment; BAL, bronchoalveolar lavage; GLPCK, benzoyl-glycyl-leucyl-phenylalanyl-chloromethylketone; HOCl, hypchlorous acid; MeO-ala-ala-prO-val-pNA, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; PPE, porcine pancreatic elastase; RNCl, *N*-chloroamines.

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analysis. Oxidized α-1-PI was prepared by incubating native α-1-PI with a 50-fold molar excess of *N*-chlorotaurine for 90 min at 37°C. *N*-chlorotaurine was synthesized by adding reagent NaOCl (Fisher Scientific Co., Fair Lawn, NJ) to a fivefold excess of taurine (Sigma Chemical Co., St. Louis, MO). The α-1-PI was then dialyzed overnight against two changes of Dulbecco's buffer (1 vol/500 vol). Pooled human AB sera was prepared as previously described (27) and stored at −70°C. The sera pool contained 2.46 mg/ml α-1-PI as determined by radial immunodiffusion assay (Calbiochem-Behring Corp.). Alveolar fluid recovered after BAL was concentrated by pressure dialysis using an Amicon chamber equipped with a YM-10 membrane (Amicon Corp., Danvers, MA). The concentrate was adjusted to an α-1-PI concentration of 15 μg/ml (~0.71 mg total protein/ml). Purified human neutrophil elastase isolated from sputum (28) was obtained from Elastin Products (Pacific, MO) and was determined to be free of cathepsin G activity. The neutrophil elastase substrate, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (MeO-ala-ala-pro-val-pNA), was obtained from Calbiochem-Behring Corp., and the neutrophil elastase inhibitor, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-chloromethylketone (AAPVCK), and the cathepsin G inhibitor, benzoyl-glycyl-leucyl-phenylalanyl-chloromethylketone (GLPCK), from Enzyme Systems Products (Livermore, CA).

Cell preparation

Human neutrophils were isolated from the venous blood of healthy donors by Ficoll-Hypaque density centrifugation followed by dextran sedimentation as previously described (29). Cells were suspended in Dulbecco's phosphate-buffered saline (M. A. Bioproducts, Walkerville, MD) supplemented with 1 mg/ml glucose adjusted to pH 7.4.

Assay of neutrophil elastase activity

Neutrophils (2.5×10^5) were incubated in the absence or presence of 30 ng of phorbol myristate acetate (PMA; Consolidated Midland Corp., Forrester NY) in 25 or 200 μl of Dulbecco's buffer for 60 min at 37°C. Other additions to the incubation mixture included bovine catalase (88,000 U/mg; Cooper Biomedical Inc., Malvern, PA), azide, methionine, purified α-1-PI, chemically oxidized α-1-PI, pooled human sera, or BAL fluid at the concentrations indicated in Results. At the end of the incubation period, 25 μg of human serum albumin (Sigma Chemical Co., St. Louis, MO) was added in a final volume of 50 μl to enhance recovery of the released proteinase (30). The cells were pelleted by brief centrifugation (500 *g* for 3 min), the supernatants were carefully removed and treated with 500 nmol of methionine to reduce residual *N*-chloroamines (31, 32). The mixtures were then assayed for elastase activity or further treated with the neutrophil elastase inhibitor, AAPVCK (33), the cathepsin G inhibitor, GLPCK (33), α-1-PI, or pooled serum for 10 min at 25°C. The chloromethylketone inhibitors were dissolved in 100% ethanol as 3 mM stock solutions. Control experiments demonstrated that, at the concentrations used, ethanol alone had no inhibitory effect on neutrophil elastase activity. All mixtures were assayed for neutrophil elastase activity with 1 mM MeO-ala-ala-pro-val-pNA (dissolved in dimethyl sulfoxide) in 0.1 M Hepes buffer at pH 7.5 containing 0.5 M NaCl and 10% dimethyl sulfoxide at 25°C (34). Elastase activity was expressed as nanomoles of substrate cleaved per hour, assuming an extinction coefficient of 8.8×10^3 M^{-1} cm^1 at 410 nm (34). In serum samples, elastase activity bound to α-2-macroglobulin was determined after free elastase was inhibited by 5.0 μg of exogenous α-1-PI (4, 35). All results are expressed as the mean±1 SEM unless indicated otherwise.

Assay of porcine pancreatic elastase inhibitory capacity of α-1-PI and serum

Varying doses of purified α-1-PI or serum were incubated with phorbol myristate acetate (PMA) stimulated neutrophils (1.25×10^5 to 2.5×10^5) reagent hypochlorous acid (HOCl), *N*-chlorotaurine, or cell-derived *N*-chloroamines for indicated periods of time in Dulbecco's buffer. *N*-chloroamines generated by neutrophils were prepared by incubating 2.5×10^6 neutrophils with 30 ng/ml PMA as previously described (32). HOCl, *N*-chlorotaurine, and endogenous *N*-chloroamines were quantitated spectrophotometrically by their ability to oxidize 5-thionitrobenzoic acid ($\Sigma_{412nm} = 1.36 \times 10^4$ M^{-1} cm^{-1}) to its disulfide derivative (29). Other additions to the incubation system included bovine catalase, superoxide dismutase (Boehringer Mannheim Biochemicals, Indianapolis, IN), azide, desferrioxamine (CIBA Pharmaceutical Co., Summit, NJ), methionine, ethanol, or dimethyl sulfoxide. In selected experiments, neutrophils (2.5×10^6) were stimulated with 30 ng/ml PMA in a final volume of 1 ml inside sealed dialysis bags (Union Carbide Co., Film Pack Division, Chicago, IL). (Before use, the dialysis tubing was first pretreated with 1 mM *N*-chlorotaurine for 24 h to remove contaminating reducing agents and then incubated for an additional 24 h in distilled water. The tubing was finally washed with Dulbecco's buffer at which time no residual oxidizing activity could be detected). The bags containing the neutrophils were then suspended in polypropylene tubes containing 100 μg of α-1-PI or 0.25% serum in a final volume of 2 ml and incubated on a rocking platform at 37°C.

At the end of the indicated incubation period, the reactions were terminated by removing an aliquot of the sample to a test tube containing 500 nmol of methionine to quench residual oxidants and the cell-free supernatant isolated by centrifugation (500 *g* × 5 min). The elastase inhibitory capacity of α-1-PI was determined with PPE (Worthington Biochemical Corp., Freehold, NJ) as previously described (26). Briefly, 1.0 μg of the elastase was mixed with known amounts of purified α-1-PI (Σ_{280nm} 1% = 5.2) for 5 min at 25°C and the residual elastase inhibitory capacity was determined spectrophotometrically with *N*-succinyl-alanyl-alanyl-alanyl p-nitroanilide (Sigma Chemical Co.). The percent loss of the elastase inhibitory capacity was calculated by comparing the ability of equal amounts of control and test samples of α-1-PI to suppress PPE activity. In 20 experiments, the calculated amount of native α-1-PI required to inhibit 1.0 μg of PPE completely was 3.40±0.36 μg (mean±1 SD) while 50 μg of the chemically oxidized α-1-PI exerted no inhibitory effect ($n = 6$).

Amino acid analysis of α-1-PI

Purified α-1-PI (50 μg) was incubated with either 1.25×10^5 resting or PMA-triggered neutrophils or 10 nmol cell-derived *N*-chloroamines for 90 min at 37°C. The amino acid analyses of the cell-free supernatants were performed as previously described (23). The conversion of methionine to methionine sulfoxide was determined according to the method of Shechter et al. (36) and the tryptophan content by fluorescence photometry (23).

SDS-PAGE analyses

SDS-PAGE analyses of the interactions of purified α-1-PI, serum or BAL with PPE, purified neutrophil elastase, or triggered neutrophils were performed as described below.

α-1-PI interactions with PPE. Native or chemically oxidized α-1-PI (25 μg) were incubated alone or with 1.25×10^5 PMA-triggered neutrophils in a final volume of 250 μl for 60 min at 37°C. The mixtures were then treated with 500 nmol methionine (10 μl), the cells were pelleted by centrifugation, and 100 μl of the supernatant containing ~10 μg of α-1-PI was removed. The native α-1-PI, neutrophil-oxidized α-1-PI, or chemically oxidized α-1-PI were then incubated alone or with 1.6 μg of PPE for 30 min at 25°C. The samples were heated at 100°C for 5 min in 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8) and analyzed by discontinuous slab gel electrophoresis according to the method of Laemmli (37) using a 3% polyacrylamide stacking gel (pH 6.8) and a 7.5% polyacrylamide resolving gel (pH 8.8). After electrophoresis, the gels were incubated in 50% trichloroacetic acid overnight and protein bands were visualized with Coomassie brilliant blue R (Sigma Chemical Co.). Molecular weight analysis was performed by comparing the migration of proteins relative to that of standards (Sigma Chemical Co.), which included carbonic anhydrase (M_r 29,000), egg albumin (M_r 45,000), bovine albumin (M_r 66,000), and phosphorylase B from rabbit muscle (M_r 97,400). Under these conditions, native and oxidized α-1-PI co-migrated with an apparent M_r of ~58,000. Similar values have been reported by others (Banda et al. [19]; personal

communications with Dr. M. J. Banda, University of California, San Francisco, and Dr. C. Glaser, Institutes of Medical Sciences, San Francisco), but differ from the known M_r for α-1-PI of 53,000 (4). To determine whether this discrepancy was due to the retarded migration of α-1-PI in gels secondary to its heavy glycosylation, the M_r of a nonglycosylated α-1-PI (a/a variant; kindly provided by Dr. J. Travis, University of Georgia, Athens) was also calculated on the basis of its migration on SDS-PAGE. In this situation, the variant migrated with the expected M_r of 46,000–47,000.

α-1-PI interactions with neutrophil elastase. Neutrophils (2.5×10^5) were incubated with PMA alone or with native or chemically preoxidized α-1-PI in the presence or absence of 5 mM methionine in a final volume of 200 μl and incubated for 60 min. The cell-free supernatants were prepared as described above. In selected experiments, native or preoxidized α-1-PI (5–10 μg) were incubated with 0.4–0.8 μg of purified neutrophil elastase for 60 min. All samples were then analyzed by SDS-PAGE and protein bands were visualized with a silver stain (Bio-Rad Laboratories, Richmond, CA).

Serum or BAL interactions with neutrophil elastase. Neutrophils (2.5×10^5) were triggered with PMA in the presence of 0.25% serum or a quantity of the BAL fluid adjusted to an equivalent α-1-PI concentration (~1.5 μg α-1-PI with a total protein of 70 μg), in a final volume of 200 μl and incubated for 60 min. Both the serum and the adjusted BAL fluids contained equivalent PPE inhibitory activity. The cell-free supernatants were subjected to SDS-PAGE, the proteins transferred from the gel to nitrocellulose as described (38), and the α-1-PI immunologically detected with highly purified rabbit antiserum to human α-1-PI (Calbiochem-Behring Corp.) as described by Towbin et al. (38). Bound antibody was then visualized with peroxidase-labeled goat anti-rabbit IgG and 4-chloro-1-napthol (Bio-Rad Laboratories).

Protein determination

Protein concentration was determined by the method of Lowry using a bovine albumin standard (39).

Results

Regulation of endogenous elastase activity released from neutrophils. PMA-triggered neutrophils (2.5×10^5) suspended in buffer alone released significant amounts of active elastase during a 60-min incubation (Table I). The released activity was almost completely inhibited by treating the cell-free supernatant with either 1.0 μg α-1-PI or the neutrophil elastase inhibitor, AAPVCK, but not the cathepsin G inhibitor, GLPCK (Table I). In order to determine the ability of the neutrophil to maintain its released elastase in an active form in the presence of an excess of α-1-PI (i.e., ≥1.0 μg, a dose able to completely inhibit all of the elastase released from the stimulated neutrophil), PMA-triggered cells were incubated with increasing concentrations of the native antiproteinase (1–100 μg/0.2 ml) for 60 min and the cell-free supernatants examined for neutrophil elastase activity. As shown in Fig. 1 *A*, 1.0 μg of α-1-PI, a dose equal to that required to inhibit all of the elastase released by 2.5×10^5 PMA-stimulated cells, was unable to control the released activity. Indeed, when the neutrophils were triggered in the presence of 5, 10, 25, or 50 μg of α-1-PI, significant quantities of free elastase could still be detected (Fig. 1 *A*). Only when the α-1-PI dose was increased to 100 μg (i.e., a 100-fold excess of the antiproteinase) was elastase activity no longer detectable (Fig. 1 *A*). Heat-denaturation of 100 μg of α-1-PI completely destroyed its inhibitory activity ($n = 2$).

In physiological fluids, neutrophil elastase can be inhibited by either α-1-PI or α-2-macroglobulin (4). Although α-1-PI is the primary regulator of elastase in serum or plasma, analyses of neutrophil elastase activity in the presence of both antiproteinases are complicated by the fact that enzyme complexed with α-2-macroglobulin retains activity towards small molecular weight substrates (4). Nonetheless, free elastase can be differentiated from α-2-macroglobulin-complexed elastase by the insensitivity of the bound enzymes's activity to α-1-PI (4, 35). Thus, neutrophils (2.5×10^5) were triggered in the presence of increasing concentrations of serum (0.125–2.5%), the serum-containing supernatants were recovered, incubated in the absence or presence of an excess of purified α-1-PI (5 μg), and then assayed for residual elastase activity. As shown in Fig. 1 *B*, both free neutrophil elastase and α-2-macroglobulin-complexed elastase could be detected in serum at concentrations as high as 1.25%. In contrast, if neutrophils were triggered in the absence of serum, all of the free elastase activity in the supernatant could be inhibited by as little as 0.125% serum (data not shown). Free neutrophil elastase activity was inconsistently maintained when 2.5×10^5 neutrophils were triggered in the presence of 2.5% serum while α-2-macroglobulin-bound elastase remained detectable (Fig. 1 *B*). Identical results were obtained if serum was replaced with autologous, heparinized plasma (data not shown). It should be noted that the final serum concentration was less important than the total amount of serum present. For example, if 2.5×10^5 cells were triggered in the presence of 5% serum in a final volume of 25 μl, they maintained 15.6±1.5% ($n = 3$) of their released elastase in an active form.

The ability of serum to suppress the released neutrophil elastase activity could be rendered even less efficient if a serum incubation mixture was exposed to a second bolus of stimulated neutrophils. In four experiments, 2.5×10^5 neutrophils stimulated in the presence of 1.25% serum for 60 min in a final volume of 200 μl maintained 1.7±0.5% of their released elastase in an active form. However, if an additional 2.5×10^5 neutrophils were added to the mixture at the end of this period and incubated

Table I. Quantitation and Characterization of Released Neutrophil Elastase Activity

Additive*	Neutrophil elastase activity§
	nmol MeO-ala-ala-pro-val pNA cleaved/h
Supernatant from resting neutrophils	11.7±1.5
Supernatant from PMA-triggered neutrophils (complete system)*	75.8±9.4
Complete system + α-1-PI (1.0 μg)‡	1.0±0.5
Complete system + AAPVCK (7.5 nmol)	1.7±0.1
Complete system + GLPCK (7.5 nmol)	58.6±10.0

* Complete system consisted of the supernatant from 2.5×10^5 neutrophils stimulated with PMA (30 ng) in a final volume of 0.2 ml for 60 min at 37°C.
‡ Supernatants were incubated with inhibitors for 10 min at 25°C.
§ Results are expressed as the mean±1 SD ($n = 4$).

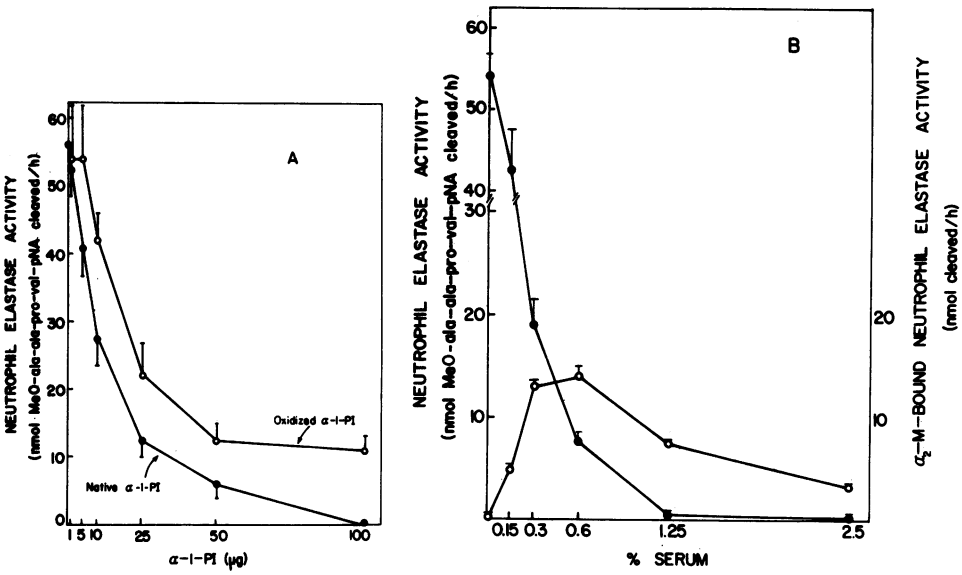

Figure 1. Neutrophil elastase activity released by neutrophils triggered in the presence of α-1-PI or serum. Neutrophils (2.5×10^5) were stimulated with PMA alone or in the presence of the indicated dose of (*A*) α-1-PI or (*B*) serum in a final volume of 200 μl, and the released elastase activity was determined after a 60-min incubation. (*A*) Neutrophils were triggered in the presence of either native α-1-PI (●) or chemically preoxidized α-1-PI (○). Neutrophil elastase activity is expressed as the mean nanomoles of MeO-ala-ala-pro-val-pNA cleaved per hour±1 SEM for 22 experiments in the absence of α-1-PI, 8 experiments for native α-1-PI at the 1.0-, 5.0-, 10-, 25-, and 50-μg doses, and 4 experiments at the 100-μg dose. The numbers of experiments performed in the presence of oxidized α-1-PI were 5, 5, 12, 5, 9, and 4 at the respective doses. Native α-1-PI exerted a significant inhibitory effect ($P \leq 0.0025$, paired Student *t* test) at all doses with the exception of the 1.0-μg dose whereas oxidized α-1-PI significantly inhibited elastase activity in the 10–100-μg dose range. (*B*) Neutrophils were triggered in the presence of serum and both free elastase activity (●) and elastase activity associated with α-2-macroglobulin (○) were determined. Results are expressed as described above. The number of experiments performed at the serum concentrations from 0.0% to 2.5% were 11, 5, 11, 10, 7, and 5, respectively.

for another 60 min, 40.4±10.6% of the released elastase activity could be detected (29.9±6.0 nmol cleaved/h) relative to cells stimulated in an identical manner in the absence of serum. Taken together, these results indicate that neutrophils triggered in the presence of quantities of either purified α-1-PI or serum in excess of those required to completely inhibit all of the released elastase still maintained a significant portion of the released proteinase in an active form.

Inactivation of PPE inhibitory capacity of α-1-PI. In order for the triggered neutrophil to maintain free elastase activity in the presence of an excess of α-1-PI, the native antiproteinase must be modified. Triggered neutrophils have been demonstrated to use oxygen metabolites to alter α-1-PI activity such that the antiproteinase is incapable of inhibiting exogenous PPE (20–22). Unlike neutrophil elastase, PPE is not complexed by oxidized α-1-PI ($k_{association} = 0\ M^{-1}\ s^{-1}$) and provides a convenient tool for monitoring the oxidation of the antiproteinase (4, 25). As shown in Fig. 2, native α-1-PI (lane *1*) formed an SDS-stable complex with PPE[2] (lane *2*), whereas α-1-PI exposed to triggered neutrophils retained its normal M_r (lane *3*), but did not complex PPE and was cleaved to a 52–53-kD fragment (lane *4*). An identical pattern was obtained after the addition of PPE to chemically oxidized α-1-PI (lane *5*). Immunoblot analyses demonstrated that α-1-PI in serum was also sensitive to oxidative inactivation (see below).

Kinetic analyses of the rate of α-1-PI oxidation revealed that at doses ≤50 μg the PPE inhibitory capacity of the native antiproteinase was completely lost by the end of the standard 60-min incubation period (Fig. 3). As the α-1-PI dose was increased to 100 μg, a concentration at which free neutrophil elastase could no longer be detected, the triggered phagocytes failed to completely oxidize the antiproteinase (Fig. 3). Thus, at doses of α-1-PI ≤ 50 μg, the time required for the complete oxidation of the antiproteinase was inversely related to the amount of neutrophil elastase activity detected (compare Figs. 1 *A* and 3). However, at higher doses, the neutrophil could significantly attenuate the antiproteinase shield, but not to a degree sufficient to allow released neutrophil elastase to remain active.

Regulation of neutrophil elastase activity by oxidized α-1-PI. Oxidation of α-1-PI leads to a complete loss in PPE inhibitory capacity, but as discussed, should only decrease the rate constant of association of the antiproteinase with neutrophil elastase (4, 25). Indeed, the complete oxidation of the native α-1-PI at the 10-, 25-, or 50-μg doses increases the calculated half-life of released neutrophil elastase to only 25, 10, and 5 s, respectively.[3] In order to assess directly the ability of the oxidized antiproteinase to regulate neutrophil elastase activity, phagocytes were triggered in the presence of increasing doses of chemically preoxidized α-1-PI (1–100 μg) and free activity was determined as described above. As shown in Fig. 1 *A*, preoxidized α-1-PI could exhibit a partial inhibitory effect, but in contrast to the calculated results, large excesses of the modified antiproteinase were unable to completely inhibit released elastase activity. At the highest dose tested, heat-inactivated oxidized α-1-PI (100 μg) had no effect on elastase activity ($n = 2$). Thus, neither an excess of native nor oxidized α-1-PI could efficiently control the elastase released from the triggered neutrophil.

Identification of the oxygen metabolites responsible for the neutrophil-mediated oxidation of α-1-PI. The oxidation of α-1-

2. Although the majority of elastase is bound by α-1-PI to form the expected complex, elastase can also nonspecifically cleave α-1-PI outside its inhibitory site to form a small quantity of an additional α-1-PI fragment that migrates with an M_r intermediate to that of native α-1-PI and the oxidized α-1-PI_f (40).

3. The half-life time of reaction between elastase and an excess of oxidized α-1-PI can be determined as $t_{0.5} = (0.693)/k_{association}(I^0)$, where the $k_{association}$ for elastase and oxidized α-1-PI is taken as $3.1 \times 10^4\ M^{-1}\ s^{-1}$ and I^0 is the concentration of the oxidized α-1-PI (25).

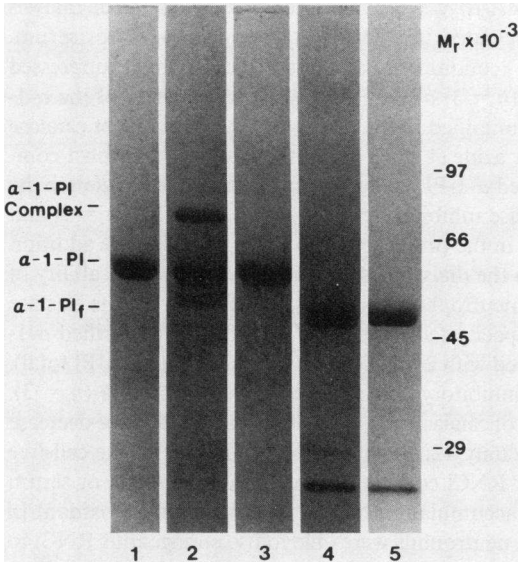

Figure 2. Analysis of the interaction of neutrophil-inactivated α-1-PI with porcine pancreatic elastase. α-1-PI (25 μg) was incubated alone or with 1.25×10^5 PMA-stimulated neutrophils for 60 min at 37°C. The preparations were treated with methionine and aliquots containing 10 μg of α-1-PI were removed. Chemically oxidized α-1-PI was prepared as described in Methods. The α-1-PI (10 μg) was then incubated with 1.6 μg of porcine pancreatic elastase for 30 min at 25°C and analyzed by SDS-PAGE as described in Methods. Lane *1*, native α-1-PI; lane *2*, native α-1-PI plus porcine pancreatic elastase; lane *3*, neutrophil-oxidized α-1-PI; lane *4*, neutrophil oxidized α-1-PI plus porcine pancreatic elastase; lane *5*, chemically oxidized α-1-PI plus porcine pancreatic elastase. The molecular weights of protein standards (see Methods) are shown to the right of the figure. The lanes shown are from a single representative gel, which was cut and rearranged for purposes of comparison.

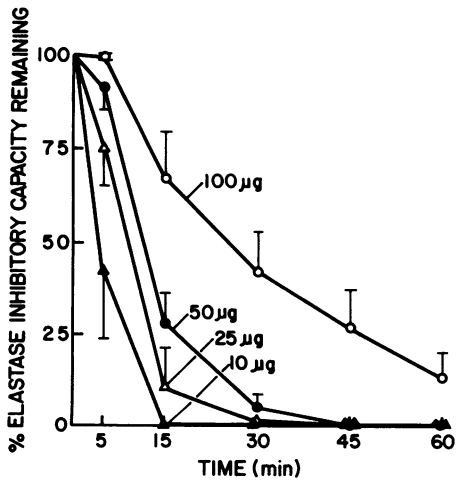

Figure 3. Rate of inactivation of α-1-PI by neutrophils as a function of the antiproteinase concentration. Neutrophils (2.5×10^5) were stimulated with PMA (30 ng) in the presence of 10 (▲), 25 (△), 50 (●), and 100 μg (○) of purified α-1-PI in a final volume of 0.2 ml and the percent pancreatic elastase inhibitory capacity remaining was determined at each time point. Results are expressed as the mean±1 SEM of three experiments.

PI consumes the pool of native antiproteinase and results in the accumulation of a modified inhibitor incapable of efficiently controlling neutrophil elastase. Superoxide anion (O_2^-), H_2O_2, the hydroxyl radical (OH·), and the myeloperoxidase system have all been implicated in the neutrophil-mediated oxidation of α-1-PI (20–24), but the identity of the final attacking specie(s) remains unclear. Thus, we examined the ability of triggered neutrophils to suppress the PPE inhibitory capacity of purified α-1-PI or serum in the presence of agents known to lower the O_2^- concentration (superoxide dismutase), degrade H_2O_2 (catalase), scavenge OH· (ethanol, dimethyl sulfoxide), chelate ferric ions (desferrioxamine), inhibit myeloperoxidase (azide), or scavenge chlorinated oxidants (methionine). As shown in Table II, only native catalase, azide, or methionine was able to protect the PPE inhibitory capacity of purified α-1-PI or serum. Thus, the generation of myeloperoxidase-derived oxidants was required for the suppression of α-1-PI activity.

Intact neutrophils use the myeloperoxidase system to generate HOCl and a complex mixture of long-lived *N*-chloroamines (RNCl) (31, 32, 41). As shown in Fig. 4, 10 nmol of reagent HOCl, *N*-chlorotaurine (the major hydrophilic RNCl generated by phagocytes; see Test et al. [32]), or cell-derived RNCl destroyed the PPE inhibitory capacity of purified α-1-PI as a function of time. In data not shown, identical results were obtained with 10 nmol of the respective oxidants and 0.25% serum. These

Table II. Role of Oxygen Metabolites in the Inactivation of the Elastase Inhibitory Capacity of α-1-PI and Serum by Triggered Neutrophils

Additive	Percent loss of elastase inhibitory capacity	
	α-1-PI (50 μg)	Serum (0.25%)
	%	%
Neutrophils + PMA (complete system)	98.7±1.1	84.6±10.6
Complete system + superoxide dismutase (10 μg)	90.6±3.7	64.6±9.5
Complete system + autoclaved superoxide dismutase	—	73.1±14.1
Complete system + catalase (10 μg)	0.9±8.7	12.9±6.3
Complete system + autoclaved catalase (10 μg)	95.3±3.0	80.9±20.8
Complete system + albumin (10 μg)	94.9±0.7	—
Complete system + azide (0.1 mM)	0.5±0.9	9.2±4.2
Complete system + dimethyl sulfoxide (50 mM)	88.6±7.3	80.8±2.4
Complete system + ethanol (40 mM)	100.3±2.5	82.1±14.5
Complete system + desferrioxamine (0.5 mM)	94.0±3.9	75.8±10.6
Complete system + methionine (5 mM)	1.0±2.0	2.8±5.2

Neutrophils (1.25×10^5) were incubated with PMA (30 ng/ml) in the presence of purified α-1-PI (50 μg) or serum (0.25%) in a final volume of 1 ml of Dulbecco's buffer at 37°C for 120 min (complete system). Reactions were terminated and the porcine pancreatic elastase inhibitory capacity of α-1-PI or serum was assayed as described. Results are expressed as the mean percent loss of α-1-PI activity±1 SD in four experiments with purified α-1-PI and seven experiments with serum.

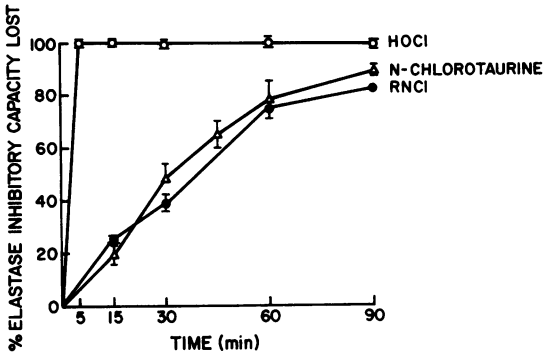

Figure 4. The inactivation of α-1-PI by HOCl, *N*-chlorotaurine, and endogenous RNCl as a function of time. Purified α-1-PI (50 μg) was incubated with 10 nmol of HOCl (○), *N*-chlorotaurine (△), or RNCl (●) in a final volume of 1 ml of Dulbecco's buffer at 37°C. Reactions were terminated and the porcine elastase inhibitory capacity of the α-1-PI was determined as described. Results are expressed as the mean percent loss of activity±1 SEM of three experiments.

results indicate that intact neutrophils can potentially use either class of chlorinated oxidant to inactivate α-1-PI. However, neutrophils normally shunt <20% of the total HOCl generated into long-lived RNCl (32, 41), and it might be expected that HOCl itself is the preferred oxidant for inactivating α-1-PI. Indeed, the addition of exogenous taurine (5 mM) to the triggered neutrophils results in the interception and trapping of almost all the generated HOCl as *N*-chlorotaurine (29) and markedly slowed (but did not inhibit) the rate of α-1-PI oxidation in the purified α-1-PI or serum systems (Fig. 5).

Triggered neutrophils may favor using HOCl to oxidize α-1-PI over short distances, but the greater stability and selectivity of the RNCl should allow the cell to attack α-1-PI over distances normally considered outside the range of phagocyte-mediated oxidative effects. In order to determine directly the ability of intact neutrophils to utilize RNCl to oxidize α-1-PI over larger

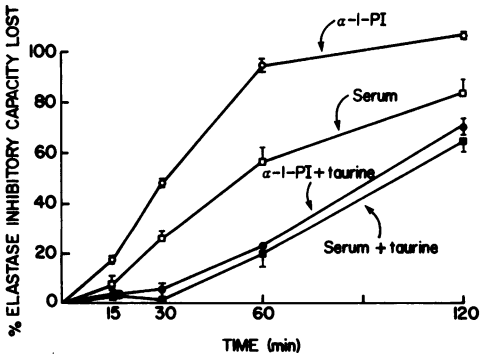

Figure 5. The effect of taurine on the inactivation of α-1-PI by PMA-triggered neutrophils as a function of time. Neutrophils (1.25×10^5) were stimulated with PMA (30 ng/ml) in the presence of purified α-1-PI (50 μg) or serum (0.25%) in a final volume of 1 ml of Dulbecco's buffer. Reactions were terminated and the α-1-PI elastase inhibitory capacity was determined as described. Neutrophils triggered with α-1-PI alone (○), with α-1-PI and 5 mM taurine (●), with serum alone (□), and with serum and 5 mM taurine (■). Results are expressed as the mean percent loss of α-1-PI activity±1 SEM in three experiments.

distances, PMA-triggered phagocytes were sealed inside dialysis bags and suspended in solutions of purified α-1-PI or serum. Under these conditions, stimulated neutrophils suppressed 87.0±14.8% (n = 3) of the PPE inhibitory capacity of the α-1-PI (100 μg) contained in the dialysate. The presence of catalase (25 μg/ml) or azide (1 mm) inside the dialysis bag almost completely blocked α-1-PI oxidation (in the presence of catalase the porcine elastase inhibitory capacity fell 6.7±11.5% [n = 3] and 0.0% [n = 2] in the presence of azide). In contrast, the addition of catalase to the dialysate alone had no effect on the ability of the triggered neutrophils to suppress α-1-PI activity (the elastase inhibitory capacity fell 93.0±6.6%, n = 3). If the purified α-1-PI was replaced with 2 ml of 0.25% serum (12.5 μg α-1-PI total), the elastase inhibitory capacity decreased 59.3±5.5% (n = 3). The addition of catalase into the dialysis bag blunted the decrease in activity to only 12.7±9.6% (n = 3). Analysis of the cell-free dialysates for RNCl content in the absence of α-1-PI or serum revealed the accumulation of 11.4±4.6 nmol of the oxidant [n = 3]).[4] Thus, neutrophils were able to use endogenous RNCl to suppress antiproteinase activity at sites distant from the cell.

α-1-PI contains critical methionine and tyrosine residues (42, 43), but the oxidative modifications underlying neutrophil-mediated inactivation have not been described. Thus, α-1-PI samples oxidized by triggered neutrophils or endogenous RNCl were examined by amino acid analysis as described in Methods. In the presence of 1.25×10^5 PMA-triggered neutrophils, the sole alteration detected in the amino acid structure of α-1-PI after a 90-min incubation was the oxidation of four of the eight methionine residues to their sulfoxide derivative (n = 3). α-1-PI incubated alone or with resting neutrophils did not contain any sulfoxide residues (n = 3). Increasing the triggered neutrophil concentration from 1.25×10^5/ml to 1×10^6/ml did not result in the oxidation of additional residues.

Incubation of α-1-PI with cell-free supernatants containing 10 nmol of the RNCl produced by PMA-triggered neutrophils also yielded a modified antiproteinase containing four methionine sulfoxide residues (n = 2). If the endogenous RNCl were first reduced back to their parent amines by the addition of an excess of methionine and then incubated with α-1-PI, no alterations in the structure of the antiproteinase were detected (n = 2). Thus, triggered neutrophils can use HOCl or RNCl to oxidize α-1-PI and in either situation, the modified antiproteinase contained four methionine sulfoxide residues.

Regulation of neutrophil elastase activity by α-1-PI and chlorinated oxidants. Because the escape of functional neutrophil elastase from α-1-PI minimally required the complete oxidation of the native antiproteinase, neutrophils should not be able to express free elastase activity if triggered in the presence of agents that are known to interfere with HOCl- or RNCl-mediated attack. As shown in Table III, neutrophils stimualted in the presence of catalase, azide, or methionine failed to express free elastase in either the α-1-PI or serum systems. In serum, the addition of these agents not only blocked the expression of free elastase activity, but also led to a decrease in the activity associated with α-2-macroglobulin. For example, in the presence of azide or methionine, the elastase activity associated with α-2-macro-

4. Although either HOCl or RNCl can oxidize 5-thio-nitrobenzoic acid, the oxidant detected in the dialysate was identified as RNCl based on the fact that it did not react with an equimolar concentration of H_2O_2 (31).

Table III. Effect of Chlorinated Oxidants on Neutrophil Elastase Activity in the Absence and Presence of α-1-PI or Serum

Additive*	Neutrophil elastase activity		
	− α-1-PI	+ α-1-PI (10 μg)	+ serum (0.5%)
	nmol MeO-ala-ala-pro-val-pNA cleaved/h		
Neutrophils + PMA (complete system)*	68.9±2.8 (14)§	38.3±4.0 (10)	6.7±1.1 (10)
Complete system + catalase (1.25 μg/ml)‡	80.0±4.6 (5)	0.0±0.0 (7)	0.1±0.2 (6)
Complete system + azide (0.1 mM)	269.3±30.0 (5)	0.0±0.0 (5)	0.1±0.2 (7)
Complete system + methionine (5 mM)	125.8±10.0 (9)	0.0±0.0 (9)	0.1±0.1 (10)

* Complete system consisted of 2.5×10^5 neutrophils triggered with PMA (30 ng) in a final volume of 0.2 ml incubated for 60 min at 37°C. ‡ If catalase was replaced with autoclaved catalase, then PMA-triggered neutrophils plus α-1-PI cleaved 31.5±3.8 nmol/h (n = 3) of the neutrophil elastase substrate. § Results are expressed as the mean±1 SEM (number of observations).

globulin in 0.5% serum fell from 10.6±0.9 nmol cleaved/h (n = 10) to 1.5±0.1 (n = 7) and 0.9±0.1 (n = 10) nmol, respectively. It should be noted that none of these agents interfered with elastase activity in the absence of α-1-PI or serum. Indeed, both azide and methionine consistently enhanced the amount of elastase activity detected in the cell-free supernatants (Table III).

Taken together, these results indicate that HOCl allows the neutrophil to subvert at least a 50-fold excess of α-1-PI (relative to the amount of α-1-PI required to inhibit the released elastase) in the purified antiproteinase system and a 10-fold excess in the serum system. A portion of the greater resistance of the serum to the HOCl-dependent expression of free elastase activity could be due to the presence of oxidizeable substrates that compete with α-1-PI for the generated HOCl. In order to determine the potential role of small molecular weight compounds, serum was first dialyzed (1/500, vol/vol) and then incubated with triggered neutrophils. However, dialyzed serum (0.125–2.5%) inhibited free elastase activity comparably to control serum (data not shown). Serum also contains large amounts of protein relative to its α-1-PI content and these molecules could potentially intercept HOCl (32). Indeed, if 2.5×10^5 neutrophils were triggered in the presence of 3.5 μg of purified α-1-PI (a dose approximately equal to that present in 0.6% pooled serum), 94.0±3.2% of the released elastase remained active, but if cells were stimulated in the presence of α-1-PI and 50 μg of human albumin (the equivalent of 0.6% pooled serum) only 32.7±4.0% (n = 4) of the released elastase activity was detected. (Albumin alone had no effect on elastase activity.) Thus, even though the ability of the neutrophil to use HOCl to inactivate α-1-PI in serum is blunted by the presence of alternate protein substrates, free elastase activity can nonetheless be maintained.

Analysis of neutrophil elastase-α-1-PI interactions by SDS-PAGE. The oxidation of α-1-PI leaves the molecule susceptible to proteolysis by PPE (24, 42) but its interactions with active neutrophil elastase are unclear. In order to determine the fate of α-1-PI incubated in the neutrophil system, 2.5×10^5 phagocytes were triggered in the absence or presence of the native antiproteinase for 60 min and the supernatants analyzed by SDS-PAGE. As shown in Fig. 6, supernatants recovered from neutrophils triggered alone revealed only a single well-defined band after visualization with silver stain (lane *1;* in data not shown this material co-migrated with purified lactoferrin) that could easily be differentiated from native α-1-PI (lane *2*). At a dose of 5.0 μg of α-1-PI, neutrophils rapidly oxidized all of the antiproteinase and the released neutrophil elastase activity was minimally suppressed (see Fig. 1 *A*). Under these conditions, no complex with neutrophil elastase could be detected after SDS-PAGE (lane *3*); in addition, a small quantity of the oxidized α-1-PI was cleaved into a closely migrating fragment (α-1-PI_f)

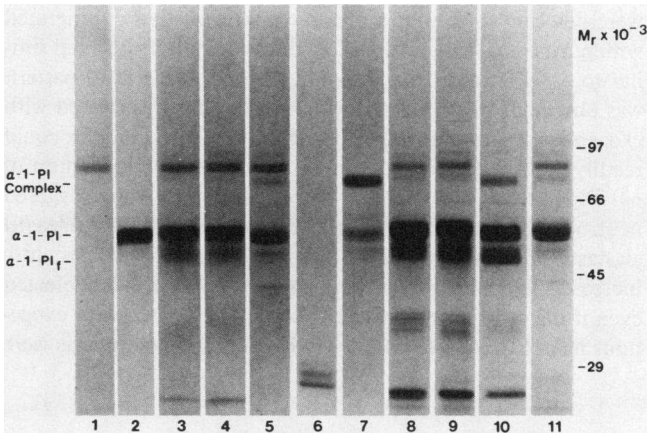

Figure 6. Analysis of the products generated during neutrophil elastase–α-1-PI interactions. Neutrophils (2.5×10^5) were incubated with PMA alone or with native or chemically oxidized α-1-PI (5.0 or 10 μg) in 0.2 ml for 60 min at 37°C. Methionine was added to each sample, cell-free supernatants were prepared as in Fig. 2, and one half of each supernatant was analyzed by SDS-PAGE as described in Methods. Lane *1*, neutrophils + PMA; lane *2*, native α-1-PI (5.0 μg); lane *3*, neutrophils + PMA + native α-1-PI (5.0 μg); lane *4*, neutrophils + PMA + chemically oxidized α-1-PI (5.0 μg); lane *5*, neutrophils + PMA + 5 mM methionine + native α-1-PI (5.0 μg); lane *6*, purified neutrophil elastase (0.8 μg); lane *7*, native α-1-PI (5.0 μg) incubated with purified neutrophil elastase (0.8 μg) for 60 min at 37°C; lane *8*, neutrophils + PMA + native α-1-PI (10 μg); lane *9*, neutrophils + PMA + chemically oxidized α-1-PI (10 μg); lane *10*, chemically oxidized α-1-PI (10 μg) incubated with purified neutrophil elastase (0.4 μg) as in lane *7;* lane *11*, neutrophils + PMA + 5 mM methionine + native α-1-PI (10 μg). Molecular weight standards are shown to the right of the figure. The lanes shown are from two representative gels, one for preparations containing 5 μg of α-1-PI and one for those containing 10 μg of α-1-PI. The lanes were cut and rearranged for purposes of comparison.

(lane *3*). An identical pattern was observed when neutrophils were triggered in the presence of chemically oxidized α-1-PI (lane *4*). In the presence of methionine, α-1-PI was protected from neutrophil-mediated oxidation (lane *5*), and a band that co-migrated with the complex formed in mixtures of purified elastase (lane *6*) and α-1-PI (lane *7*) could be detected. As the α-1-PI dose was raised to 10 μg, increased inhibition of the elastase activity released by triggered neutrophils was observed (see Fig. 1 *A*) and in this case, SDS-PAGE of the supernatant revealed both α-1-PI–neutrophil elastase complex formation and the degradation of the α-1-PI (lane *8*). A similar spectrum of products was obtained after the incubation of chemically preoxidized α-1-PI with either triggered neutrophils (lane *9*) or purified sputum elastase (lane *10*). Although complexes of neutrophil elastase and oxidized α-1-PI are less stable than those formed with native antiproteinase, a portion of these complexes remained detectable (e.g., see lane *10*). As expected, neutrophils triggered in the presence of methionine and 10 μg of α-1-PI proteolyzed only small amounts of the protected antiproteinase (lane *11*).

In order to determine the course of interactions between neutrophil elastase and α-1-PI in serum, neutrophils were triggered as described above except that the purified antiproteinase was replaced with serum (0.25% final). After a 60-min incubation, supernatants were electrophoresed and transferred to nitrocellulose, and the α-1-PI was localized with highly purified antisera (see Methods for details). As shown in Fig. 7, two bands were detected in serum; a major component that co-migrated with purified α-1-PI and a minor component that appeared similar to α-1-PI_f (lane *1;* in data not shown, an identical pattern was obtained with fresh plasma). If the serum was treated with exogenous neutrophil elastase, the elastase–α-1-PI complex could readily be detected along with an increase in the formation of α-1-PI_f (lane *2*). In contrast, serum incubated with triggered neutrophils was unable to complex the endogenously released elastase and the intensity of α-1-PI_f was slightly, but consistently increased (lane *3*). In addition, complex could not be detected even if the cell-free supernatant was supplemented with exogenous neutrophil elastase (lane *4*). However, if neutrophils were triggered in the presence of azide (lane *5*) or methionine (lane *6*), serum α-1-PI was protected from oxidative inactivation, the released elastase was complexed and a portion of the α-1-PI was fragmented to yield a pattern identical to that found in mixtures of serum and purified neutrophil elastase (see lane *2*). The immunologic identification of purified α-1-PI and its complex with purified elastase are shown in lanes *7* and *8* for comparison.

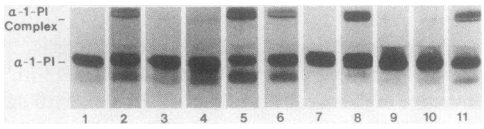

Figure 7. Immunoblot analysis of α-1-PI status in mixtures of triggered neutrophils and serum or BAL fluid. Neutrophils (2.5×10^5) were incubated with PMA and 0.25% serum or BAL fluid concentrate adjusted to an equivalent α-1-PI concentration as described in Methods. The samples were incubated for 60 min at 37°C in a final volume of 200 μl and prepared as described in Methods. Lane *1*, serum alone; lane *2*, serum incubated with 0.2 μg of purified neutrophil elastase; lane *3*, neutrophils + PMA + serum; lane *4*, supernatant from neutrophils + PMA + serum treated with 0.2 μg of purified neutrophil elastase for 10 min at 25°C; lane *5*, neutrophils + PMA + azide (0.1 mM) + serum; lane *6*, neutrophils + PMA + methionine (5 mM) + serum; lane *7*, 5 μg of purified α-1-PI alone; lane *8*, 5 μg of purified α-1-PI + 0.8 μg of purified neutrophil elastase; lane *9*, BAL fluid alone; lane *10*, neutrophils + PMA + BAL fluid; lane *11*, neutrophils + PMA + methionine (5 mM) + BAL fluid. The lanes shown are from a single representative gel of three performed. The lanes were cut and rearranged for purposes of comparison.

Recent attention has focused on the detection of free neutrophil elastase activity in BAL obtained from patients with inflammatory lung disorders (5–7). Thus, we sought to determine whether the oxidative inactivation of α-1-PI and accumulation of free elastase could also occur in mixtures of neutrophils triggered in the presence of BAL fluid. If BAL concentrate from a normal individual was adjusted to an α-1-PI concentration equal to that of 0.25% serum (see Methods) it failed to control the elastase activity released by 2.5×10^5 PMA-triggered neutrophils (85.4% of the released elastase was detected). Because normal BAL fluid contains almost no α-2-macroglobulin (44), bound elastase activity was not detected under these conditions. As expected, if neutrophils were triggered in BAL fluid in the presence of methionine (5 mM), no free elastase activity could be detected (0.0 nmol substrate cleaved/h). In a representative experiment of three performed, immunoblot analyses of the BAL fluid (lane *9*) revealed a pattern identical to that observed in serum; i.e., neutrophil elastase-α-1-PI complexes were not detected in mixtures of BAL and triggered neutrophils (lane *10*) unless the α-1-PI was protected from inactivation by exogenous methionine (lane *11*).

Discussion

The detection of active neutrophil elastase and modified α-1-PI in tissues and fluids recovered from inflammatory sites is thought to represent a critical component in the pathogenesis of tissue damage (5–16). Neutrophil elastase can directly effect coagulation, fibrinolytic, kallikrein, immunoglobulin, and complement-dependent pathways, alter cellular functions of the endothelium, platelets, and leukocytes, and degrade connective tissue components (for review and recent examples see References 3 and 45–51). Free elastase can only be detected in the presence of α-1-PI if there is an excess of proteinase or if the antiproteinase function has been modified. Recent attention has focused on the potential ability of macrophages or neutrophils to alter α-1-PI activity in a manner that would allow neutrophil elastase to accumulate. Purified macrophage proteinases can fragment α-1-PI (17, 18) and Banda et al. (19) have recently demonstrated that rabbit alveolar macrophages use an uncharacterized proteinase to inactivate the PPE inhibitory capacity of human α-1-PI in a serum-free, model system. These authors postulated that the detection of large amounts of proteolyzed α-1-PI in fluids recovered from inflammatory sites might reflect the operation of a similar process in vivo (19). In contrast, other evidence supports a more direct role for the neutrophil in the regulation of α-1-PI activity. Several reports have either detected free elastase in association with oxidized α-1-PI (6, 7) or oxidized α-1-PI alone in fluids recovered from inflamed lungs or joints (52, 53). Although many studies have assumed that the oxidation of α-1-PI would allow for the detection of free neutrophil elastase activity in vivo, it is important to note that there was no experimental evidence to support this contention. Almost all studies

of α-1-PI function after its oxidation in vitro or in vivo have been limited to determinations of the ability of the antiproteinase to inhibit exogenous porcine pancreatic elastase (5–7, 20–22). Oxidized α-1-PI is completely unable to inhibit PPE, but retains considerable inhibitory activity for neutrophil elastase (3, 25). Indeed, the association rate constant for the interaction of PPE and native α-1-PI is only three times greater than that for neutrophil elastase and oxidized α-1-PI (25). Based on this information, the oxidation of α-1-PI should have slowed the rate of elastase inactivation, but not allowed free elastase to accumulate.

In this study we have demonstrated that neutrophils triggered in the presence of a relative excess of purified α-1-PI, serum or normal BAL fluid can maintain significant amounts of their released elastase in an active state. In the purified α-1-PI system, the amount of free elastase detected was partially regulated by the initial α-1-PI concentration, its rate of oxidation and the inhibitory potential of the accumulating oxidized antiproteinase. Together, the native and oxidized α-1-PI did not exert an appreciable inhibitory effect on the released elastase activity (i.e., ≥50%) until their net concentration was in 10-fold excess of that required to inhibit the activity present in cell-free supernatants. Even when the α-1-PI dose was 50 times higher than the expected inhibitory dose, ~10% of the released neutrophil elastase activity could be detected. Our data also indicate that α-1-PI oxidized by the neutrophil or chemical oxidants could significantly inhibit neutrophil elastase activity, however, not as effectively as predicted from its rate constant of association. The reasons for this discrepancy are unclear, but the determination of the rate constant of association was performed under conditions much different from those employed in our cell system,[5] and with a modified α-1-PI that contained only two methionine sulfoxide residues (25). Nonetheless, it is known that complexes of neutrophil elastase and oxidized α-1-PI have an increased dissociation constant (54), that these complexes are not stable during gel filtration chromatography (55) and that the oxidized antiproteinase is susceptible to cleavage by neutrophil elastase (see below). Along similar lines, Beatty et al. (54) recently reported that the preincubation of purified neutrophil elastase with an equimolar quantity of chemically oxidized α-1-PI failed to inhibit the elastolytic activity of the proteinase (54). We conclude that even large excesses of oxidized α-1-PI cannot completely inhibit neutrophil elastase activity in an intact cell system.

The demonstration that neutrophils can maintain and accumulate released elastase in an active form in the presence of excess α-1-PI should not be confused with recent studies designed to examine the competitive interactions among neutrophil elastase, α-1-PI, and a connective tissue substrate. Four groups have examined the ability of neutrophils to proteolyze connective tissue components in the presence of α-1-PI or serum, and all have stressed the critical role of the neutrophil-substrate interface for the detection of elastase activity (56–59). Campbell et al. (56) proposed that triggered neutrophils degraded a fibronectin substratum by releasing elastase into a sequestered site that excluded the fluid phase α-1-PI. Along similar lines Chapman and Stone (57) reported that resting neutrophils inefficiently solubilized elastin in the presence of serum. In contrast, we and others demonstrated that neutrophils enhanced their proteolytic potential by oxidatively protecting released elastase from α-1-PI inactivation (58, 59). In each of these systems, the detection of elastase-mediated damage in the presence of a competitive substrate (i.e., fibronectin, elastin, or basement membrane-associated proteins) demonstrated that elastase had bound to a connective tissue target before it was inhibited by native or oxidized α-1-PI. If elastase must be released in a sequestered, α-1-PI free environment (56, 57), then any proteinase that leaked into the bulk phase would be rapidly complexed by native α-1-PI. Alternatively, oxidative inactivation of α-1-PI was felt to increase the half-life of elastase only long enough to allow it to bind to the connective tissue substratum (58, 59). Thus, these models did not predict or provide an explanation for the processes that allowed free neutrophil elastase to accumulate in fluids recovered from inflammatory sites. Our data clearly indicate that triggered neutrophils can directly maintain released elastase in an active state even in the absence of sequestered sites or competing substrates and that proteolytic damage will not be limited to the neutrophil-substratum interface.

The elastase released from triggered neutrophils could also be detected in a more physiologic serum system. Although these experiments were limited to dilute suspensions of serum, several factors should be considered. First, only small numbers of triggered neutrophils were used (125,000–250,000 cells) and the incubation periods were limited to 60 min. In additional experiments, higher serum concentrations could be used if the number of neutrophils was increased, while longer incubation periods allowed for the accumulation of greater amounts of elastase activity as additional proteinase was slowly released by the damaged cells (unpublished observation). Second, the serum concentration was less important than the total quantity of serum added. As demonstrated, neutrophils triggered in the presence of 5% serum in 25 μl maintained free elastase activity as well as cells stimulated in ~0.6% serum in 200 μl. We consider it unlikely that small numbers of neutrophils could maintain active elastase in a nonsequestered site in the presence of large volumes of plasma or serum (e.g., the vascular bed), but neutrophils that have infiltrated interstitial or tissue sites would be faced with only limited quantities of α-1-PI. Third, the exposure of larger amounts of physiologic fluids to multiple waves of triggered neutrophils can also subvert the antiproteinase shield. Even when neutrophils maintained only small amounts of their released elastase in an active state, this finding indicated that all of the native α-1-PI must have been oxidized. Thus, after the addition of a second bolus of neutrophils, only oxidized α-1-PI remained to regulate elastase activity inefficiently. Finally, the significance of even small amounts of free elastase activity should not be underestimated. Neutrophil elastase is an extremely stable enzyme that is able to cleave susceptible substrates for weeks (60) and once bound, the proteinase displays increased resistance to inactivation by native or oxidized α-1-PI (54, 61, 62). The accumulation of free elastase activity in the serum system was also associated with an increase in elastase activity bound to α-2-macroglobulin. α-1-PI and α-2-macroglobulin have similar rate constants of association for neutrophil elastase, but α-1-PI is present at a sevenfold higher molar concentration (63). In the presence of triggered neutrophils, the oxidation of α-1-PI depressed its activity and increased the opportunity for α-2-macroglobulin, an oxidant-resistant antiproteinase (22), to complex the released elastase. α-2-Macroglobulin physically entraps enzymatically active

5. Rate constants of association between purified human neutrophil elastase and native or chemically oxidized α-1-PI were measured indirectly by allowing elastase and bovine chymotrypsin to compete for binding with the antiproteinase during a 1-min incubation period (25).

elastase and sterically blocks the proteolytic activity of elastase towards all substrates over 8,500 D, including α-1-PI (64). Complexes of α-2-macroglobulin and elastase are rapidly cleared from the circulation, but Stone and co-workers (64, 65) have reported that complexes trapped in connective tissues can propogate additional damage after the slow release of active elastase from degraded α-2-macroglobulin. Thus, the oxidatively linked shunting of elastase from α-1-PI to α-2-macroglobulin may mediate either anti- or proinflammatory effects depending on the rate of clearance of the complex.

The detection of free elastase activity in the presence of α-1-PI or serum was completely dependent on the inactivation of the antiproteinase by chlorinated oxidants. Neutrophils use the myeloperoxidase system to simultaneously generate both HOCl and RNCl (29, 31, 32, 41) and either of these oxidants had sufficient oxidizing potential to inactivate α-1-PI. Neutrophils preferentially used the more reactive HOCl (or a chlorinated oxidant with similar reactivity) to attack α-1-PI over short distances while the more selective RNCl allowed oxidation to occur at distances normally considered outside the range of these phagocyte-mediated effects. Amino acid analyses of α-1-PI that was inactivated by triggered neutrophils or RNCl revealed the presence of four methionine sulfoxide residues per α-1-PI molecule. These results contrast with earlier in vitro studies that reported the oxidation of only two methionine residues in α-1-PI that had been exposed to increasing concentrations of H_2O_2 and myeloperoxidase (23, 24) or large molar excesses of the synthetic chloroamine, *N*-chlorosuccinimide (42). The reasons for these differences have not been directly examined, but our findings are in complete agreement with reports describing the detection of four methionine sulfoxide residues per α-1-PI molecule in fluids recovered from smokers' lungs or rheumatoid joints (52, 53).

If neutrophils were prevented from generating HOCl (e.g., catalase or azide) or if the generated oxidants were reduced by exogenous methionine, free elastase did not accumulate in the presence of α-1-PI, serum, or BAL fluid and the elastase activity associated with α-2-macroglobulin in serum decreased by ~90%. The failure to detect free elastase occurred despite the fact that these interventions actually increased the amount of proteinase activity recovered into the supernatant. Apparently, neutrophil elastase is sensitive to oxidative inactivation in a manner analogous to that described for other lysosomal enzymes (66, 67). Voetman et al. (66) first reported that a series of lysosomal enzymes are inactivated by oxidants generated within the phagosome. In our system, the ability of the small molecular weight inhibitors, azide and methionine, to increase dramatically elastase activity coupled with the inability of catalase to increase activity as effectively also suggests a sequestered site of inactivation.

The ability of an excess of methionine to protect α-1-PI from oxidative inactivation has led Banda et al. (19) to propose that it might act as an efficient, endogenous antioxidant in vivo. In their recent study, these investigators reviewed reports that used methionine in concentrations ranging from 4 to 100 μM to inhibit neutrophil-mediated oxidation of α-1-PI or *N*-formyl-methionyl-leucyl-phenylalanine (19). Based on the fact that the mean serum concentration of free methionine is 23 μM and that PMA-triggered alveolar macrophages did not oxidatively inactivate α-1-PI in culture media that contained methionine, they concluded that oxidants may only play a limited role in regulating the antiproteinase under physiologic conditions (19). However, the inability of macrophages to oxidize α-1-PI is not unexpected and is probably independent of the methionine concentration. Macrophages contain little if any myeloperoxidase (68, 69), and the ability of the phagocyte to generate alternate oxidants that are capable of inactivating α-1-PI is unclear. Even if macrophages could generate chlorinated oxidants, the quantity of methionine required to inhibit myeloperoxidase-mediated effects is not fixed, but rather it is dependent on the number of cells used and the amounts of HOCl generated. For example, 1 ml of 23 μM methionine (i.e., 23 nmol) could be completely oxidized to its sulfoxide derivative by 23 nmol of HOCl or RNCl, a quantity generated by $\sim 4 \times 10^5$ PMA-triggered neutrophils (29, 32). Thus, neutrophils triggered in a localized environment would rapidly deplete the pool of free methionine and replace it with the relatively unreactive sulfoxide. In our study, methionine efficiently blocked α-1-PI oxidation, but neutrophils were always triggered in the presence of large excesses of the thioether relative to the amount of HOCl or RNCl generated. Plasma proteins also contain methionine residues, but it is interesting that small numbers of triggered neutrophils completely oxidized α-1-PI in serum or BAL fluids despite the fact that the antiproteinase represents only 2–3% of the total protein in either millieu. It appears that the oxidizeable methionine residues in α-1-PI are situated at sites that are preferentially accessible to the fluid-phase oxidants (70).

In association with the demonstration that oxidized α-1-PI and free neutrophil elastase can be detected in vivo, analyses of the recovered α-1-PI on SDS-PAGE have revealed the presence of a fragment with an apparent M_r of ~5 kD less than the native antiproteinase (6, 7, 71). Cochrane et al. (7) demonstrated that an identical fragment could also be generated if porcine pancreatic elastase was added to BAL fluid that contained α-1-PI that had previously undergone oxidation in vivo. However, the ability of intact neutrophils to fragment α-1-PI directly has not been previously described. In our studies with purified α-1-PI, triggered neutrophils cooperatively used chlorinated oxidants and free elastase to cleave the antiproteinase into a fragment similar to that detected in vivo. In the serum and BAL fluid systems, the effect of the neutrophil on endogenous α-1-PI was complicated by the presence of an additional band that co-migrated with the expected α-1-PI fragment. We have not analyzed this material, but is was also detected in fresh plasma (data not shown) and has been previously described in normal BAL fluids (71). Nonetheless, the native α-1-PI was oxidatively inactivated in either milieu and was incapable of complexing the elastase released from the triggered neutrophils unless the antiproteinase was protected from inactivation. In contrast to the results obtained with purified α-1-PI, the free elastase did not extensively degrade the oxidized antiproteinase during the standard 60-min period in serum or BAL fluid. Presumably, less elastase was available for the proteolysis of the modified antiproteinase owing to the multiplicity of competitive serum protein substrates (e.g., see References 46–48). Finally, it is interesting that in both the serum and BAL fluid systems, the greatest increase in the formation of the α-1-PI fragment occurred when the antiproteinase was protected from oxidative inactivation and the released elastase was complexed (identical results were also obtained with mixtures of serum and purified elastase, see Fig. 7, lane *2*). Stockley and Afford (71, 72) also reported that an α-1-PI fragment was generated after the addition of neutrophil elastase to

purified α-1-PI or normal plasma. They concluded that proteolyzed α-1-PI can be generated during the slow dissociation of elastase-inhibitor complexes (71, 72). Taken together, these data indicate that triggered neutrophils can mediate α-1-PI proteolysis, but oxidative inactivation of the antiproteinase is not a required prerequisite.

In summary, we have demonstrated that human neutrophils triggered in the presence of excess α-1-PI, serum, or BAL fluids can maintain a portion of their released elastase in an active form by attacking the antiproteinase with chlorinated oxidants. The identification of free elastase activity, oxidized α-1-PI, and antiproteinase fragments closely mimics recent analyses of the neutrophil elastase and α-1-PI status in tissue fluids recovered from pathologic sites. Although other oxidant sources (i.e., hyperoxia, cigarette smoke, or environmental pollutants; e.g., see References 8, 73, and 74) and other proteinases (leukocyte or bacterial; references 17–19 and 75) may also contribute to this scheme, it seems clear that triggered neutrophils alone can directly mediate all of these effects. The ability of the neutrophil to release active elastase in the presence of α-1-PI could be useful in physiologic events such as fibrinolysis, wound healing, or host defense, but under pathological conditions, the indiscriminant release of unregulated elastase could exert powerful and long-lasting proinflammatory effects (5–16). Finally, these data lend support to our premise that chlorinated oxidants may play a more general role in controlling proteinase activity (76). In addition to regulating elastase activity, we have recently demonstrated that neutrophil collagenase (77) and gelatinase (78), two metalloproteinases that are stored in latent forms, are activated via HOCl-dependent processes. We conclude that chlorinated oxidants may initiate and propogate specific tissue degradation in a manner that would not be predicted on the basis of their own reactivity, but rather by the actions of the affected proteinases.

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