

# Regulation of Alpha<sub>1</sub> Proteinase Inhibitor Function by Rabbit Alveolar Macrophages

## Evidence for Proteolytic Rather than Oxidative Inactivation

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### Abstract

Rabbit alveolar macrophages were cultured in an environment conducive to the secretion of both reactive oxygen and proteinases, so that the relative importance of proteolytic and oxidative inactivation of  $\alpha_1$ -proteinase inhibitor by alveolar macrophages could be evaluated. The inactivation of  $\alpha_1$ -proteinase inhibitor was proportional to its proteolysis, and there was no detectable inactivation in the absence of proteolysis. Although the live macrophages were capable of secreting reactive oxygen, they did not inactivate  $\alpha_1$ -proteinase inhibitor by oxidation. The inactivation of  $\alpha_1$ -proteinase inhibitor by proteolysis was proportional to the secretion of elastolytic activity by the alveolar macrophages. The inability of the alveolar macrophages to oxidize  $\alpha_1$ -proteinase inhibitor was attributed to the methionine in the macrophages, in secreted proteins, and in the culture medium competing for oxidants. The data suggest that proteolytic inactivation of  $\alpha_1$ -proteinase inhibitor may be important in vivo and that the methionine concentration in vivo may protect  $\alpha_1$ -proteinase inhibitor from significant oxidative inactivation.

### Introduction

Alveolar macrophages are the most frequently encountered phagocytes in the pulmonary alveolar space. In addition to their role as scavengers, macrophages are potent secretory cells that can act as regulators of their microenvironment (1, 2). Their secretions include the metalloproteinases collagenase (3) and elastase (4, 5), which can destroy the integrity of the elastin connective tissue matrix that maintains the alveolar microenvironment. Degradation of elastin is typical of chronic inflammatory diseases such as emphysema. Under normal conditions, the elastin matrix is protected from the proteolytic activity of granulocyte elastase, a serine proteinase (6, 7), by a critical balance of this proteinase to its major alveolar inhibitor,  $\alpha_1$ -proteinase inhibitor ( $\alpha_1$ PI) (8, 9). It has been suggested that chronic inactivation of  $\alpha_1$ PI will predispose an individual to degenerative lung diseases (10–13). One means of inactivation

is by oxidation of the reactive site methionine residue of  $\alpha_1$ PI (14). In the lungs such oxidation may be the result of the inhalation of cigarette smoke (10–12, 14–17) or the action of reactive oxygen of cellular origin (13, 18–20). Inactivation can also result from the proteolysis of  $\alpha_1$ PI by bacterial proteinases (21, 22), by thiol proteinases (23), by macrophage elastase, a metalloproteinase (24), or possibly by other metalloproteinases secreted by macrophages. Because macrophage elastase is increased by inflammatory stimuli (1, 2, 4), alveolar macrophages may, during chronic inflammation, reduce the amount of active  $\alpha_1$ PI by proteolysis.

Because alveolar macrophages can produce both reactive oxygen (25–27) and macrophage elastase, it is possible that they inactivate  $\alpha_1$ PI by two different mechanisms. The relative importance of oxidative attenuation vs. proteolytic inactivation has not been determined. In this report we describe the ability of live alveolar macrophages and their secretions to inactivate  $\alpha_1$ PI, and we evaluate the means by which that inactivation takes place.

### Methods

**Rabbit alveolar macrophages (RAMs).** Elicited RAMs were harvested from female New Zealand White rabbits 2 wk after intravenous injection of 0.1 ml of Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY). Resident alveolar macrophages were harvested from untreated animals. Rabbits were killed by intravenous injection of sodium pentobarbital (Diabutal; Diamond Laboratories, Inc., Des Moines, IA). The lungs were removed and repeatedly washed with sterile 0.15 M NaCl. The collected cells were washed and resuspended in serum-free Dulbecco's modified Eagle's medium (DME) (Tissue Culture Facility, University of California, San Francisco, CA) supplemented with 0.2% lactalbumin hydrolysate (LH) (Difco Laboratories, Detroit, MI), and with penicillin-streptomycin. Cells were cultured at  $2 \times 10^6$  cells/ml. After 2 to 4 h of culture the nonadherent cells were removed by repeated washing with sterile 0.15 M NaCl at 37°C. The medium was replaced with fresh methionine-free DME with or without 2  $\mu$ M colchicine (Aldrich Chemical Co., Milwaukee, WI). Cells were cultured for 48 h before assays were started. At the start of the assay period cells were washed and fresh medium was added to the cells. Fresh medium was added to control wells without cells. Test reagents were added to appropriate wells from 10 or 100 times stocks to give final concentrations of 50 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, MO), 200 U/ml superoxide dismutase, 2,000 U/ml catalase, or 200  $\mu$ g/ml  $\alpha_1$ PI. Incubation of the experimental cultures and controls was then continued and medium was sampled from 0 to 52 h after addition of the test reagents. The sampled medium was frozen at  $-20^\circ\text{C}$  until assayed.

**Enzymes and inhibitors.** Mouse macrophage elastase was purified as previously described (5). Porcine pancreatic elastase, bovine liver catalase, bovine blood superoxide dismutase, horseradish peroxidase, and partially purified human  $\alpha_1$ PI were purchased from Sigma Chemical Co. The partially purified  $\alpha_1$ PI was  $\sim 70\%$   $\alpha_1$ PI, with albumin as the major contaminant. Serum albumin is a poor substrate for mouse macrophage elastase (5). The concentrations of  $\alpha_1$ PI given in the

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1. *Abbreviations used in this paper:*  $\alpha_1$ PI,  $\alpha_1$ -proteinase inhibitor; DME, Dulbecco's modified Eagle's medium; LH, lactalbumin hydrolysate; PMN, polymorphonuclear leukocytes; RAM(s), rabbit alveolar macrophages; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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Results section express the exact amount of  $\alpha_1$ PI and do not include the albumin contaminant. Homogeneous human  $\alpha_1$ PI, a gift of C. Glaser (Institutes of Medical Sciences, San Francisco, CA), was purified by published methods (28); on SDS-polyacrylamide gels it migrated at  $M_r$  58,000 either as a single band or as a double band (an artifact of Laemmli system gels) (24).

Homogeneous  $\alpha_1$ PI was radioiodinated by the method of Bolton and Hunter (29), which labels the lysine residues so as to avoid inactivation of  $\alpha_1$ PI by oxidation of the methionine residues.  $^{125}$ I-labeled Bolton-Hunter reagent was purchased from New England Nuclear, Boston, MA. Specific activity of  $^{125}$ I-labeled  $\alpha_1$ PI varied from 0.1 to 0.2  $\mu$ Ci/nmol.  $^{125}$ I-labeled  $\alpha_1$ PI was stored at 4°C until used. It was added as a tracer to 2 mg/ml of nonradioactive  $\alpha_1$ PI (Sigma Chemical Co.) to give up to  $4.7 \times 10^7$  dpm/ml.

**Cell-free oxidation of  $\alpha_1$ PI.** Oxidized  $\alpha_1$ PI was prepared by mixing 200  $\mu$ g/ml of  $\alpha_1$ PI in DME-LH or methionine-free DME with sufficient *N*-chlorosuccinimide (Sigma Chemical Co.) to give molar ratios of 2:1, 10:1, and 20:1 (*N*-chlorosuccinimide/ $\alpha_1$ PI). The mixtures were incubated at room temperature for 20 min, and reactions were stopped by the addition of 10 mM methionine. The inhibitory capacity of oxidized  $\alpha_1$ PI was determined as described subsequently.

**Elastase assay.** Elastolytic activity was measured by determining the amount of soluble radioactivity released from insoluble [ $^3$ H]elastin in the presence of SDS as previously described (5). 1 U of elastase activity was defined as the solubilization of 1.0  $\mu$ g of elastin/h at 37°C.

**Assay for inhibitory capacity of  $\alpha_1$ PI.** The inhibitory capacity of  $\alpha_1$ PI was determined by measuring the residual activity of pancreatic elastase incubated with  $\alpha_1$ PI. Pancreatic elastase activity was distinguished from macrophage elastase activity by determining the rate of cleavage of succinyl-trialanyl-paranitroanilide, a substrate for pancreatic elastase (30) that is not degraded by macrophage elastase. The amount of  $\alpha_1$ PI in each reaction mixture was verified by determining the amount of  $^{125}$ I radioactivity. Each reaction mixture was normalized to the 0 h medium control, which was considered 100% inhibition of pancreatic elastase.

**Reactive oxygen production.** Cellular production of  $O_2^-$  was assayed by determining the TPA-inducible, superoxide dismutase-suppressible reduction of ferricytochrome C (31). The rate of  $H_2O_2$  production was assayed by the peroxidase-mediated extinction of scopoletin fluorescence (32).

**Electrophoresis.** SDS-polyacrylamide gradient gel electrophoresis was performed as described previously (24). After electrophoresis, the protein bands were stained with 0.05% Coomassie Brilliant Blue R250 dissolved in 20% (vol/vol) methanol. Radiolabeled proteins were located by autoradiography on Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY) (33).

## Results

**Elastase secretion by alveolar macrophages.** In a previous study (34) we showed that RAMs secrete an elastase similar to the elastase secreted by mouse macrophages (5). Both have the characteristics of a metalloproteinase and are not inhibited by  $\alpha_1$ PI (4, 5, 24). RAMs that had been cultured for 48 h and then placed in fresh culture medium secreted elastase during the next 52 h (Fig. 1). Elicited RAMs expressed nearly twice the amount of elastase activity as resident RAMs; the conditioned medium from elicited RAMs at 19 h and from resident RAMs at 52 h contained similar amounts of elastase activity. The secretion of elastase activity by elicited RAMs was further enhanced to threefold over that of resident RAMs by treatment with TPA. Unlike mouse macrophages, RAMs did not secrete more elastase activity in the presence of 2.0  $\mu$ M colchicine (data not shown).

**Proteolysis of  $\alpha_1$ PI by RAMs.** To study the potential proteolysis of  $\alpha_1$ PI by RAMs, we added  $^{125}$ I-labeled  $\alpha_1$ PI to

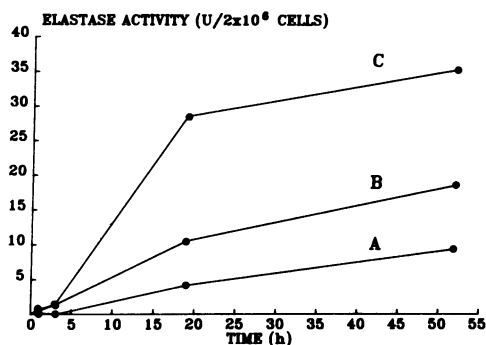


Figure 1. Secretion of elastase activity by RAMs. RAMs were placed in fresh medium after 48 h in culture, and the elastase activity secreted into the fresh medium was assayed as described in Methods. The time axis represents hours of culture in fresh medium. Activities are from: A, resident RAMs; B, elicited RAMs; C, elicited RAMs treated with TPA.

the culture medium of RAMs that had been placed in culture 48 h previously. The molecular weight of the  $\alpha_1$ PI was then monitored by autoradiography of SDS-polyacrylamide gradient electrophoretic gels (Fig. 2). Proteolysis by resident RAMs was first detected after 52 h of incubation. However, the proteolysis of  $\alpha_1$ PI after 19 h of incubation with elicited RAMs was equivalent to that of the resident RAMs at 52 h. When TPA-treated elicited RAMs were examined, the proteolysis of  $\alpha_1$ PI after 19 h of incubation was greater than that seen after any amount of incubation with untreated resident or elicited RAMs. These findings are similar to those in the experiment to detect secretion of elastase (Fig. 1). Therefore, the proteolysis of  $\alpha_1$ PI by living RAMs was proportional to the amount of elastase activity secreted into the medium. It is interesting to note that the  $^{125}$ I-labeled  $\alpha_1$ PI did not accumulate in the cells during these experiments.

**Inactivation of  $\alpha_1$ PI by RAMs.** In previous work we showed that the proteolysis of  $\alpha_1$ PI by macrophage elastase resulted in the inactivation of  $\alpha_1$ PI (24). Macrophages can secrete reactive oxygen species that could inactivate  $\alpha_1$ PI in the absence of proteolysis. Therefore, the same conditioned media that were monitored for proteolysis of  $\alpha_1$ PI by SDS-polyacrylamide gel electrophoresis (Fig. 2) were assayed for inactivation of  $\alpha_1$ PI by testing their ability to inactivate pancreatic elastase (Fig. 3). The inhibitory capacity of  $\alpha_1$ PI was decreased in proportion to its proteolytic degradation, and inactivation by other means, such as oxidation, was not detected. Inactivation of  $\alpha_1$ PI by

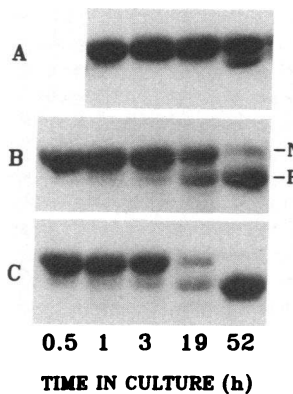


Figure 2. Proteolysis of  $\alpha_1$ PI by RAMs. Autoradiographs of SDS-polyacrylamide electrophoretic gels. The positions of intact native  $\alpha_1$ PI (N),  $M_r$  58,000, and of proteolytically degraded  $\alpha_1$ PI (P),  $M_r$  54,000, are indicated. Each lane is labeled with time in culture as measured in Fig. 1. Panels depict proteolysis by: A, resident RAMs; B, elicited RAMs; C, elicited RAMs treated with TPA.

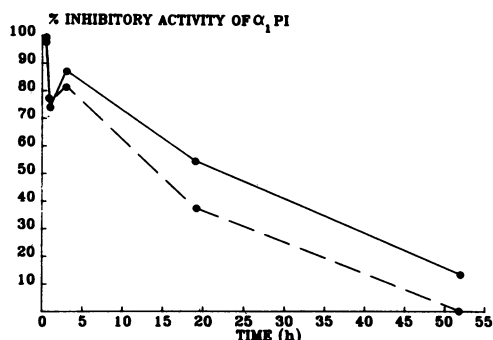


Figure 3. Inactivation of  $\alpha_1$ PI by RAMs. Samples of the same culture medium depicted in Fig. 2 (B and C) were assayed for the ability of  $\alpha_1$ PI to inhibit porcine pancreatic elastase, a serine proteinase, as described in Methods. Time in culture was measured as in Fig. 1. (—) Medium from elicited RAMs; (---) medium from elicited RAMs treated with TPA.

the oxidation of the active site methionine residue is rapid; had it occurred, it would have been detected with the pancreatic elastase inactivation assay at the earlier time points, even though inactivated  $\alpha_1$ PI would have appeared identical to native  $\alpha_1$ PI on SDS-polyacrylamide electrophoretic gels. These data show that, in medium conditioned by RAMs,  $\alpha_1$ PI remains active unless it is degraded by a macrophage proteinase. Our findings show, therefore, that not only the proteolysis but also the inactivation of  $\alpha_1$ PI by RAMs was proportional to the amount of elastase activity secreted into the medium.

**Secretion of reactive oxygen species by RAMs.** The data presented thus far are consistent with the hypothesis that proteolysis by macrophage elastase, rather than oxidative attenuation, is the means by which RAMs inactivate  $\alpha_1$ PI. To establish that the RAMs used in these experiments were secreting reactive oxygen, we determined the TPA-inducible, superoxide dismutase-suppressible reduction of ferricytochrome-C in RAM cultures. As indicated in Fig. 4, RAMs actively secreted  $O_2^-$  in both the standard serum-free culture medium (DME-LH) and in the methionine-free DME medium used in this study. Similar results were obtained for  $H_2O_2$  secretion, as monitored by the peroxidase-mediated extinction of scopoletin fluorescence (data not shown). Therefore, the inability to detect oxidized  $\alpha_1$ PI in the culture medium could not be attributed to the inability of RAMs to secrete reactive oxygen.

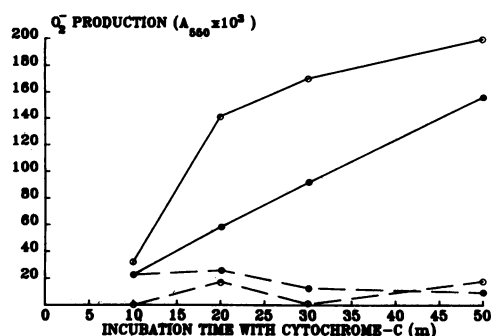


Figure 4. Production of reactive oxygen by RAMs.  $O_2^-$  production was measured by determining the oxidation of ferricytochrome-C as a change in absorbance at 550 nm. ○, RAMs cultured in DME-LH; ●, RAMs cultured in methionine-free DME; (—), TPA-treated RAMs; (---), superoxide dismutase-treated RAMs.

**Oxidation of  $\alpha_1$ PI in cell culture medium.** Chemical oxidation of  $\alpha_1$ PI by incubation with increasing concentrations of *N*-chlorosuccinimide was carried out in the various tissue culture media that were used in this study (Fig. 5). These experiments established that the methionine present in unsupplemented DME at 200  $\mu$ M competed very effectively with the methionine in the reactive site of  $\alpha_1$ PI and thus protected it from oxidation. In medium supplemented with 0.2% LH, the  $\alpha_1$ PI was completely protected from oxidative attenuation. Therefore, macrophages that are maintained in standard culture medium would not be able to oxidize  $\alpha_1$ PI because of the quenching of the  $O_2^-$  by soluble methionine or by proteins containing an accessible methionine residue.

## Discussion

Our data lead us to conclude that, in an environment favorable to the production of both oxygen radicals and macrophage elastase, macrophages inactivate  $\alpha_1$ PI by proteolysis rather than by oxidation.

Because of the central role of  $\alpha_1$ PI in controlling the proteinase balance, the regulation of  $\alpha_1$ PI function has been the subject of several investigations. In some of those studies the  $\alpha_1$ PI activity in cell-free bronchoalveolar lavage fluids of cigarette smokers and nonsmokers was compared to determine if the oxidants in cigarette smoke could inactivate  $\alpha_1$ PI (11–16, 35). The findings in these studies varied from no inactivation (15, 16) to slight inactivation (35) and to reduction in  $\alpha_1$ PI function (11–14). Although various explanations have been offered to account for the discrepancy in the observations (35), no consensus has yet been reached. Oxidized  $\alpha_1$ PI has also been detected in rheumatoid synovial fluid (36), and oxidation was considered the prominent means of inactivation of  $\alpha_1$ PI in studies of patients with adult respiratory distress syndrome (37–39). In one study of nine patients, attempts to demonstrate restoration of  $\alpha_1$ PI function by treatment with reductants were correlated with SDS-gel electrophoretic analysis of the size of  $\alpha_1$ PI (39). Attempts at reduction of the inactive  $\alpha_1$ PI restored no more than 25% of total  $\alpha_1$ PI function in five of those patients. All five patients had proteolyzed  $\alpha_1$ PI, and four of the five had very few or no  $\alpha_1$ PI-proteinase complexes. One interpretation of this study could be that the  $\alpha_1$ PI was not

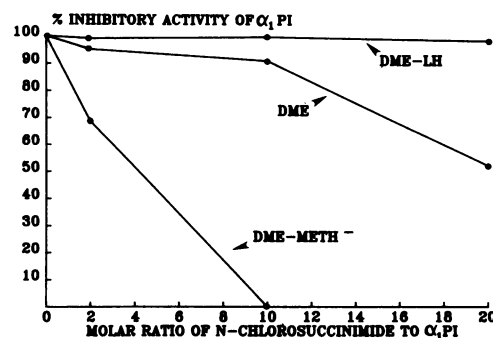


Figure 5. Oxidation of  $\alpha_1$ PI in cell culture medium. The effect of cell culture medium on the cell-free oxidation and inactivation of  $\alpha_1$ PI was determined by measuring the ability of  $\alpha_1$ PI treated with *N*-chlorosuccinimide to inhibit pancreatic elastase activity. The concentration of  $\alpha_1$ PI in these experiments remained constant and equal to that added to RAM cultures (200  $\mu$ g/ml).

oxidized but directly proteolyzed by a proteinase that does not form an inhibitory complex with  $\alpha_1$ PI.

In all of these studies, either the oxidant was of extracellular origin or the cellular source was not known. In the present study we examined the inactivation of  $\alpha_1$ PI by isolated lung cells that were maintained in tissue culture. We specifically sought to determine if RAMs inactivate  $\alpha_1$ PI and, if so, whether by proteolysis or oxidation. Our data show that exogenous  $\alpha_1$ PI was inactivated by RAMs and that reactive oxygen of cellular origin, although present, did not play a role in that inactivation. The inactivation of  $\alpha_1$ PI could be completely accounted for by the secretion of proteolytic activity by the RAMs even though they actively produced reactive oxygen.

We interpret the inability of RAMs to oxidize  $\alpha_1$ PI to be due to the protection afforded by the free methionine residues found in the culture medium (Fig. 5) and to the methionine residues incorporated into the secretory products of the RAM. It has been suggested that other inflammatory cells may be capable of inactivating  $\alpha_1$ PI by oxidation. Polymorphonuclear leukocytes (PMN) and monocytes that are cultured in methionine-free Hanks' balanced salt solution supplemented with 4% serum oxidatively inactivate  $\alpha_1$ PI (17). However, the resulting methionine concentration is  $<1 \mu\text{M}$ , a permissive concentration for oxidation. A methionine concentration of  $100 \mu\text{M}$  has been reported to prevent 100% of the oxidation of  $\alpha_1$ PI by both the purified myeloperoxidase system and activated PMN, whereas  $18 \mu\text{M}$  methionine prevents 50% of the oxidation by the PMN system (15). Other work has shown that proteinases from live activated PMN cultured in RPMI-1640, which contains  $100 \mu\text{M}$  methionine, do not oxidize endogenous  $\alpha_1$ PI (20). This study is in agreement with the data presented here for alveolar macrophages and with other studies on PMN inactivation of  $\alpha_1$ PI (15).

As oxidation is not likely to be limited to  $\alpha_1$ PI, methionine should protect other compounds from oxidation as well. The oxidative inactivation of the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine has been blocked by methionine concentrations as low as  $4 \mu\text{M}$  (40). Such concentrations can be attained in vivo. The mean serum concentration of free methionine is  $23 \mu\text{M}$  (41). This may be a low estimate of the total methionine available in tissue because methionine is found in most proteins, some of which may be available for oxidation.

The active site methionine residue in  $\alpha_1$ PI should be no more susceptible to oxidation in vivo than any other accessible methionine residue; therefore, significant concentrations of free methionine sulfoxide as well as other oxidized proteins should be detected along with oxidized  $\alpha_1$ PI. Oxidation would be a widespread event and not limited to  $\alpha_1$ PI. In contrast, proteolytic inactivation can be limited to a specific set of substrates recognized by the proteinase. In the case of RAMs, the inactivation of  $\alpha_1$ PI and the resulting tissue damage may be localized to the site of inflammation where the macrophage secretes its proteinases.

In a cell-free system, neutrophil myeloperoxidase has been shown to inactivate  $\alpha_1$ PI (19). In that study, peroxidase from homogenates of human alveolar macrophages was also shown to inactivate  $\alpha_1$ PI. This discrepancy in the ability of macrophages to oxidize  $\alpha_1$ PI is most likely due to differences in the experimental systems. In the present study, the ability of live macrophages to inactivate  $\alpha_1$ PI via a secretory product was

investigated. In the peroxidase study, extracts of macrophage homogenates were used to carry out inactivation experiments in a relatively methionine-free environment, and intracellular rather than secretory functions were examined. Therefore, those data do not contradict our findings that suggest that proteolytic activity, rather than oxidizing capacity, is the primary regulator of  $\alpha_1$ PI in live alveolar macrophages.

We used elastinolytic activity as an indicator of the proteolytic activity secreted by RAMs. Although the inactivation and proteolysis of  $\alpha_1$ PI paralleled the secretion of elastinolytic activity, we cannot exclude the possibility that other nonserine proteinases secreted by the macrophages may also have been involved. These data do show that live alveolar macrophages can secrete proteinases that directly degrade elastin as well as degrade and inactivate  $\alpha_1$ PI. The secretion of such a proteinase or combination of proteinases would establish an environment that would permit serine elastases from other cells, such as neutrophil elastase, to degrade elastin unchecked. Such a scenario could occur in the absence of any oxidation of  $\alpha_1$ PI.

Macrophages have been reported to synthesize and secrete  $\alpha_1$ PI (42, 43). The amount of  $\alpha_1$ PI produced by macrophages has not been clearly established, but human monocytes and breast-milk macrophages produce significant quantities of  $\alpha_1$ PI mRNA and active  $\alpha_1$ PI (44). In this study we did not address the fate of any  $\alpha_1$ PI that may have been secreted by macrophages. Because  $\alpha_1$ PI never forms an inhibitory complex with macrophage elastase (24), or any other metallo or thiol proteinase, the secretion of  $\alpha_1$ PI by macrophages would not interfere with the activity of macrophage elastase or invalidate the observations described in this report. Indeed, proteolytic inactivation of the  $\alpha_1$ PI secreted by macrophages may be a means of autoregulating the inhibitory capacity of  $\alpha_1$ PI.

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