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Potential Mediator of Inflammation: *PHAGOCYTE-DERIVED OXIDANTS SUPPRESS THE ELASTASE-INHIBITORY CAPACITY OF ALPHA$_1$-PROTEINASE INHIBITOR IN VITRO*

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PHAGOCYTE-DERIVED OXIDANTS SUPPRESS THE ELASTASE-INHIBITORY CAPACITY OF ALPHA$_1$-PROTEINASE INHIBITOR IN VITRO

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ABSTRACT Human polymorphonuclear leukocytes, monocytes, or pulmonary alveolar macrophages, stimulated in vitro by phorbol myristate acetate (PMA), released reactive oxygen species able to suppress the elastase inhibitory capacity (EIC) of human serum. Immunoelectrophoresis using antibodies against α_1-proteinase inhibitor (α_1-Pi) and elastase showed that inactivation of α_1-Pi was responsible for the decreased serum EIC. Treatment of phagocyte-inactivated serum with a reducing agent (dithiothreitol) resulted in significant recovery of EIC, suggesting that α_1-Pi had been oxidatively inactivated. Serum EIC was partially protected by superoxide dismutase or catalase. Hydrogen peroxide alone had no effect on serum EIC. Thus, neither H_2O_2 nor O_2^- alone, but a product of the two, may have oxidatively inactivated α_1-Pi. In support of the foregoing, neutrophils or monocytes from a patient with chronic granulomatous disease failed to produce detectable levels of O_2^- after incubation with PMA. These cells also failed to suppress serum EIC. In the case of PMA-stimulated polymorphonuclear leukocytes or monocytes, extracellular myeloperoxidase may have also played a role in α_1-Pi inactivation since serum EIC was partly protected by azide, cyanide, or the depletion of extracellular chloride. Indeed, in a cell-free system consisting of purified myeloperoxidase, a glucose oxidase–H_2O_2-generating system, and Cl^-, the EIC of human serum or purified α_1-Pi could also be suppressed. Omission of any single reactant prevented this effect, as did NaN_3 or catalase, suggesting that enzymatically active myeloperoxidase and H_2O_2 were necessary. Immunoelectrophoresis of myeloperoxidase-inactivated serum showed that, as before, inactivation of α_1-Pi was responsible for the decreased EIC. Treating myeloperoxidase-inactivated serum with dithiothreitol led to significant recovery of EIC, again suggesting that oxidative inactivation of α_1-Pi had occurred. Oxidative inactivation of α_1-Pi in the microenvironment of inflammatory cells, at sites of acute or chronic inflammation, may allow proteases released from these cells to damage adjacent connective tissue components more readily.

INTRODUCTION

Human leukocytes contain potentially harmful substances within their cytoplasmic granules, including proteases capable of degrading connective tissue structures (1). These proteases include human polymorphonuclear leukocyte (PMN)[1] elastase (2) and cathepsin-G (3). PMN accumulate at sites of inflammation and these enzymes may escape to the outside of the cell with subsequent damage to surrounding connective tissue structures (4). In addition, mononuclear phagocytes are able to secrete an elastase (5). There is, however, a system of antiproteases in the circulation and tissue fluids that inactivates proteases released from PMN (6). α_1-proteinase inhibitor (α_1-Pi) is an important component of the antiprotease system in man and is responsible for >90% of the elastase inhibitory capacity (EIC) of normal human serum (6). The local balance between released protease and tissue antiprotease is considered important in determining whether inflammation will damage connective tissue (6).

Recent reports have shown that oxidation inactivates α_1-Pi (7–10). This is attributed to oxidation of methionine residues in or near the active site of α_1-Pi (7–10). Because leukocytes produce and release several reac-

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[1] *Abbreviations used in this paper*: α_1-Pi, α_1-proteinase inhibitor; CAT, catalase; CGD, chronic granulomatous disease; DTT, dithiothreitol; EIC, elastase inhibitory capacity; HBSSG, glucose in Hank's balanced salt solution; HICAT, heat-inactivated CAT; IEP, immunoelectrophoresis; MPO, myeloperoxidase; MNC, monocyte; PAM, pulmonary alveolar macrophage; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase.

tive oxygen species during phagocytosis (11), the local balance between protease and antiprotease may be further disrupted during inflammation by the oxidative inactivation of α_1-Pi in the microenvironment of these cells. In this event, tissue components adjacent to leukocytes at sites of inflammation would be even more susceptible to damage by proteases simultaneously released from the phagocytes.

In support of this hypothesis, we recently reported (12) that human PMN, stimulated by opsonized antigen-antibody complexes bound to the outer surface of a dialysis membrane, produce oxidants able to inactivate α_1-Pi inside the dialysis bag. We also presented indirect evidence showing that inactivation of α_1-Pi in this system was dependent on hydrogen peroxide (H_2O_2), superoxide (O_2^-), and myeloperoxidase (MPO). In the same study, a cell-free superoxide-generating system was shown to inactivate α_1-Pi (12). A closely related study (13) demonstrated that purified MPO can inactivate α_1-Pi in the presence of H_2O_2 and halide ions at acid pH.

We have extended our studies of these reactions to include nonphagocytic stimuli, such as membrane-perturbing agents, and cells of chronic as well as acute inflammation. The present report demonstrates that human PMN, monocytes (MNC) and pulmonary alveolar macrophages (PAM) stimulated in vitro by phorbol myristate acetate (PMA) are capable of oxidatively inactivating α_1-Pi. Our evidence shows that inactivation of α_1-Pi depends, at least in part, on O_2^-, H_2O_2, and MPO. MNC and PMN from a patient with chronic granulomatous disease, in which generation of O_2^- and H_2O_2 is depressed, failed to inactivate α_1-Pi when exposed to PMA. Finally, purified MPO, in a cell-free system, inactivated α_1-Pi at physiological pH in a reaction dependent on H_2O_2 and Cl^-.

METHODS

Preparation of leukocytes. PMN and MNC were prepared from the peripheral blood of healthy volunteers and from a patient with chronic granulomatous disease by a modification of the technique of Böyum (14). Cells were suspended briefly in Hank's balanced salt solution (pH 7.8) containing 0.2% glucose (HBSSG) in place of diluted plasma, and centrifuged at 160 *g* for 20 min in the cold to separate the leukocytes from the bulk of platelets, before Ficoll-Hypaque separation. Typical PMN preparations contained 97–99% PMN and <3% mononuclear cells, as determined by differential counting in a hemocytometer. Cells were suspended in HBSSG (pH 7.8) containing 4% (vol/vol) human serum or other reactants to give a final concentration of 1.6×10^6 PMN/ml. Over 95% of the PMN excluded trypan blue before and after the experimental period.

The mononuclear cells were removed from the HBSS/Ficoll–Hypaque interface and washed three times with 15 vol of HBSSG (pH 7.8). The percent MNC in washed preparations was estimated by differential counting in a hemocytometer and 1.5×10^6 MNC suspended in HBSSG were poured into a 35-mm diameter tissue-culture dish. After incubation for 2.5 h at 37°C, the culture dishes were washed with HBSSG to remove nonadherent cells. Typical adherent MNC preparations contained >94% MNC and <1.4% granulocytes (the remainder being lymphocytes). Sufficient HBSSG containing 4% (vol/vol) human serum was added to each culture dish to give a final concentration of 1.6×10^6 adherent MNC/ml. Over 94% of the MNC excluded trypan blue before and after the experimental period.

Bronchoscopic, subsegmental, sterile-saline lavage was performed by standard procedure on two male, noninfected, former smokers undergoing diagnostic fiberoptic bronchoscopy. Only cells from normal lobes were used. Recovered cells were separated from lavage fluid by centrifugation at 200 *g* for 15 min and washed three times with HBSSG (pH 7.8). The percent macrophages in washed preparations was estimated by differential counting in a hemocytometer, and 6×10^4 macrophages suspended in HBSSG were poured into a 6 mm diameter tissue-culture dish. After incubation for 2.5 h at 37°C, the culture dishes were rinsed vigorously with HBSSG to remove nonadherent cells. Typical adherent macrophage preparations contained >95% macrophages and <1% PMN (the remainder being lymphocytes). Sufficient HBSSG containing 4% (vol/vol) human serum was added to each culture dish to give a final concentration of 1.6×10^6 adherent PAM/ml. Over 92% of the PAM excluded trypan blue before and after the experimental period.

Source of elastase inhibitor. Dilutions of a standard human serum containing 2.2 mg/ml of α_1-Pi (determined by radial immunodiffusion) or purified α_1-Pi (a gift of Dr. J. Travis, University of Georgia) were used as sources of elastase inhibitor.

Assay of EIC. Each reaction was run in triplicate. 50 μl of a solution to be tested for EIC was mixed with 20 μl of 0.3 μg/μl porcine pancreatic elastase (Elastin Products Co., St. Louis, Mo.). After incubation for 5 min at 37°C, the elastase activity was measured using succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (Sigma Chemical Co., St. Louis, Mo.) as substrate (15).

Immunoelectrophoresis. 50 μl of a solution to be analyzed by immunoelectrophoresis (IEP) was incubated with 5 μl of 0.6 μg/μl porcine pancreatic elastase (molar ratio of active elastase:α_1-Pi = 1) for 5 min at 37°C. 10 μl of the reaction mixture was analyzed by IEP as previously described (8) using antiserum against human α_1-Pi (Accurate Chemical & Scientific Corp., Hicksville, N. Y.) and antiserum against porcine pancreatic elastase (prepared by us).

Special reagents and assays. Leukocyte O_2^- production was measured by a modification of the method of McCord and Fridovich (16). Each reaction was run in triplicate. Leukocytes were incubated with 80 μM cytochrome *c*, after which the samples were centrifuged at 200 *g* for 15 min in the cold to remove all cells. The absorbance of the supernate at 550 nm was measured. Some reaction mixtures contained superoxide dismutase (SOD) (400 U/ml). Controls containing all reagents except leukocytes were incubated in parallel and used as blanks.

PMA (a gift of Dr. W. Troll, New York University School of Medicine) was stored at −80°C as a stock solution (1 mg/ml) in methyl alcohol. SOD (Type 1, Sigma Chemical Co., St. Louis, Mo.) was also assayed according to McCord and Fridovich (16). Both native and heat-inactivated SOD were diluted in 2 mM Na_2EDTA and then dialyzed against buffer before use to remove free metals. Catalase (CAT) (C-40, Sigma Chemical Co., St. Louis, Mo.) was freed from contaminating SOD by repeated washings through an XM100A diaflo ultrafiltration membrane (Amicon Corp., Lexington, Mass.). Our preparations of SOD and CAT were found to be free of cross-contamination. Glucose oxidase (Type V) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Purified canine MPO

was a gift of Dr. J. Schultz (Papanicolaou Cancer Research Institute). Peroxidase activity was determined by monitoring the peroxidase-catalyzed oxidation of *o*-dianisidine (Sigma Chemical Co., St. Louis, Mo.) (17). β-glucuronidase activity was measured by the method of Bretz and Baggiolini (17). Heat-inactivated MPO, CAT, and SOD were autoclaved for 20 min at 120°C and then centrifuged at 10,000 *g* for 15 min.

RESULTS

Inactivation of elastase inhibitor by PMA-stimulated phagocytes. To study the effects upon α_1-Pi of oxidants generated from phagocytes during the "respiratory burst," independently of the release of azurophilic granule proteases (which are also capable of inactivating the inhibitor), we used PMA to stimulate PMN respiration under conditions in which azurophil degranulation is minimal (18, 19). PMA was also used to stimulate the respiratory burst in MNC and PAM (18). Human PMN, MNC, or PAM were incubated with PMA in the presence of either human serum inhibitors or cytochrome *c*, unless otherwise noted. After 30 min at 37°C in a gyrorotary water bath, the cells were removed by centrifugation and the supernates were assayed for either EIC or reduced cytochrome *c* as described in Methods. Superoxide generation was observed in the medium surrounding PMA stimulated phagocytes (Table I). The EIC of serum or purified α_1-Pi incubated with PMA-stimulated phagocytes was partly suppressed (Table I). Neither PMA alone nor phagocytes without PMA significantly affected serum EIC (Table I). Control experiments ruled out activation of elastase by products of stimulated phagocytes (Table I).

TABLE I

Suppression of the EIC of Human Serum or α_1-Pi by Stimulated Human Phagocytes

Incubation mixture*					Assay results‡	
Cell type	CH_3OH	PMA	Serum	α_1-Pi	O_2^-§	EIC‖
–	–	–	–	–	NT¶	0**
–	+	+	–	–	NT	0**
–	–	–	+	–	NT	100
–	+	+	+	–	NT	98±9
PMN	+	–	+	–	0	97±9
PMN	+	+	+	–	100	45±6
PMN	+	+	–	–	93±5	0**
MNC	+	–	+	–	8±3	95±5
MNC	+	+	+	–	100	52±8
MNC	+	+	–	–	95±9	0**
PAM	+	–	+	–	24±14	90±11
PAM	+	+	+	–	100	55±5
PAM	+	+	–	–	92±9	0**
PMN(CGD)	+	+	+	–	0	97±9
PMN(CGD)	+	+	–	–	NT	0**
MNC(CGD)	+	+	+	–	0	96±8
MNC(CGD)	+	+	–	–	NT	0**
–	–	–	–	+	NT	100
–	+	+	–	+	NT	98±7
PMN	+	–	–	+	0	95±5
PMN	+	+	–	+	100	30±5

* Concentrations: cells = 1.6×10^6/ml in all cases; PMA = 1.5 ng/ml with PMN or MNC and 100 ng/ml with PAM; CH_3OH (PMA solvent) = 0.03% (vol/vol); human serum = 4% (vol/vol); pure α_1-Pi = 0.15 μg/μl. The buffer was HBSSG (pH 7.8).

‡ The results are expressed as the mean of three experiments ± 1 SEM.

§ The results given in the table are expressed as a percent of the amount of O_2^- generated by PMA-stimulated leukocytes of each cell type. 100% corresponds to 37±7 nM $O_2^-/1.6 \times 10^6$ PMN, 22±5 nM $O_2^-/1.6 \times 10^6$ MNC, and 18±4 nM $O_2^-/1.6 \times 10^6$ PAM.

‖ EIC = elastase standard − elastase + serum (or α_1-Pi)/elastase standard × 100. The results given in the table are expressed as a percent of the EIC of serum or purified α_1-Pi incubated with buffer alone.

¶ NT, not tested.

** Controls for the effects of all reagents on the activity of elastase. EIC = 0 represents 100% enzyme activity.

The EIC of serum remained unchanged when fresh serum was incubated with supernates, obtained by prior exposure of PMN, MNC, or PAM to PMA in serum-free HBSSG, and then centrifuged. This suggests that stable products (such as proteases) released from PMA-stimulated phagocytes were not responsible for suppression of serum EIC in the experiments summarized in Table I. Other evidence against α_1-Pi-inactivation by azurophil granule proteases includes (*a*) failure to detect α_1-Pi-protease complexes by crossed immunoelectrophoretic analysis (data not shown), (*b*) failure of chronic granulomatous disease PMN or MNC (in which degranulation is normal but generation of oxidants is defective) to inactive α_1-Pi (see below) and (*c*), β-glucuronidase and MPO detection in amounts $\leq 1\%$ of their total cellular content in supernates from PMA-stimulated PMN and MNC. Although some enzyme may have adhered to cell membranes during centrifugation, this last observation implies that significant bulk release of azurophil granule enzymes had not taken place after PMA stimulation. The foregoing results suggest that bulk release of azurophil granule proteases was not responsible for the observed suppression of serum EIC. Whereas proteases inactivate the inhibitor stoichiometrically (13), azurophil granule MPO is able to catalytically inactivate α_1-Pi. Evidence follows to suggest that small quantities of released MPO, acting catalytically, may play a role in the suppression of serum EIC by PMA-stimulated phagocytes.

IEP analysis of serum inactivated by PMA-stimulated phagocytes. To demonstrate that the decrease in serum EIC was due to inactivation of α_1-Pi, serum samples whose EIC had been previously suppressed by PMA-stimulated phagocytes were incubated with elastase and then analyzed by IEP as described in Methods. As shown in Fig. 1A, α_1-Pi present in control serum was able to complex all of the added elastase (Fig. 1A). In contrast, α_1-Pi in serum incubated with PMA-stimulated PMN did not complex all of the added elastase and free elastase could be detected migrating toward the cathode (Fig. 1B). Similar results were obtained with serum previously incubated with PMA-stimulated MNC or PAM (results not shown). As illustrated in Fig. 1, inactivation of α_1-Pi did not change its immunoreactivity or native electrophoretic mobility. Destruction or endocytosis of α_1-Pi by the leukocytes was not responsible for the decreased EIC, since rocket IEP showed no change in α_1-Pi concentration after incubation with PMA-stimulated cells (not shown).

Reactivation of serum EIC after incubation with PMA-stimulated phagocytes by treatment with dithiothreitol. In view of the wide range of oxidant species generated by PMA-stimulated phagocytes (11) and the known sensitivity of α_1-Pi to oxidative inactivation (7–10), it seemed likely that oxidative mechanisms were responsible for inactivation of α_1-Pi. To test this,

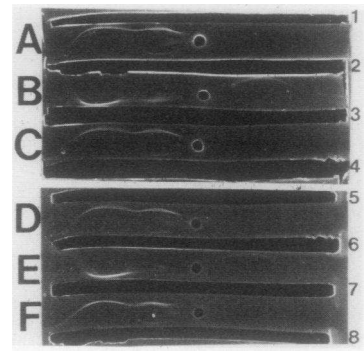

FIGURE 1 Immunoelectrophoretic analysis of mixtures of elastase and serum under various experimental conditions. Troughs 1, 3, 5, and 7 contain rabbit antiserum to human α_1-Pi. Troughs 2, 4, 6, and 8 contain rabbit antiserum to porcine pancreatic elastase. Anode to the left, cathode to the right. (A) Elastase added to control serum that had been incubated with unstimulated PMN. Note the presence of free α_1-Pi migrating toward the anode, followed by complexes of α_1-Pi and elastase. No free elastase was detected. (B) Elastase added to serum that had been incubated with PMA-stimulated PMN. Note increased free α_1-Pi, decreased α_1-Pi : elastase complexes and uncomplexed elastase migrating toward the cathode. (C) Elastase added to serum that had been previously treated as in B, followed by treatment with DTT (see text). In contrast to B, no free elastase was detected and increased α_1-Pi-elastase complexes were present. (D) Elastase added to control serum that had been previously incubated with glucose oxidase + Cl^-. Note the presence of α_1-Pi-elastase complexes. No free elastase was detected. (E) Elastase added to serum that had been previously incubated with MPO + glucose oxidase + Cl^-. Note decreased α_1-Pi-elastase complexes and uncomplexed elastase migrating toward the cathode. (F) Elastase added to serum that had been previously treated as in E, followed by treatment with DTT (see text). In contrast to E, no free elastase was detected and increased α_1-Pi-elastase complexes were present. In A and B the conditions and concentrations are as described in Table I. In D and E the conditions and concentrations are as described in Table IV.

samples of serum, inactivated by PMA-stimulated phagocytes (PMN, MNC, or PAM), were dialyzed against a reducing agent (5 mM dithiothreitol) (DTT) in 0.05 M phosphate buffer (pH 8.1) for 24 h in the cold. This was followed by 3 h of dialysis against fresh reagent at 37°C. As shown in Table II, treatment with DTT led to significant recovery of EIC in serum that had been incubated with PMA-stimulated phagocytes. DTT treatment had no effect on control serum or elastase alone (Table II). Reactivation of α_1-Pi by DTT was confirmed by IEP analysis (Fig. 1C).

Protection of serum EIC from inactivation by PMA-stimulated phagocytes. Human MNC and neutrophils contain MPO within their azurophilic lysosomal granules (20). MPO plus a source of H_2O_2 and an oxidizable halide cofactor can catalyze a wide range of oxidation and halogenation reactions which are thought to mediate the microbicidal activity of the MPO system (21). Although PMA stimulates phagocyte respiration (in-

TABLE II
Reactivation of Serum EIC After Incubation with PMA-stimulated Phagocytes by Treatment with DTT

Incubation mixture*				Post-incubation treatment	Assay results‡
Cell type	CH_3OH	PMA	Serum	DTT§	EIC
PMN	+	+	+	−	44±8
PMN	+	+	+	+	88±7
PMN	+	−	+	+	94±5
MNC	+	+	+	−	50±7
MNC	+	+	+	+	84±7
MNC	+	−	+	+	96±6
PAM	+	+	+	−	56±6
PAM	+	+	+	+	89±8
PAM	+	−	+	+	101±8
−	+	+	+	−	97±9
−	+	+	+	+	96±8
−	+	+	−	−	0‖
−	+	+	−	+	0‖

* All concentrations and conditions are as described in Table I.
‡ The results are expressed as the mean of three experiments ±1 SEM. EIC as defined in Table I.
§ DTT = 5 mM in 0.05 M phosphate buffer (pH 8.1).
‖ Controls for the effects of all reagents on the activity of elastase. EIC = 0 represents 100% enzyme activity.

cluding H_2O_2 production), it induces only minimal azurophil degranulation (18, 19). Since MPO is capable of catalytically inactivating α_1-Pi, small quantities of this enzyme (released following PMA stimulation), combined with H_2O_2 generated by the respiratory burst, and extracellular halide could have helped oxidize serum inhibitors in our experiments. This would be consistent with a previous report implicating extracellular MPO activity in the tumoricidal action of PMA-stimulated PMN (22). We tested this possibility and were able to demonstrate that 0.3 mM NaN_3 (an inhibitor of heme enzymes such as MPO) partially protected serum EIC from inactivation by PMA-stimulated PMN and MNC, without affecting O_2^- production (Table III). Higher concentrations of azide (up to 4 mM) did not result in increased protection; although serum EIC was protected by concentrations of azide down to 0.06 mM (data not shown). In addition, 0.5 mM KCN (also an inhibitor of heme enzymes), protected serum EIC from inactivation by PMA-stimulated PMN (Table III). CAT, which decreases available H_2O_2, essentially completely protected serum EIC (Table III). Heat-inactivated CAT (HICAT) did not protect serum EIC (Table III). CAT does not affect elastase alone (Table III). However, 75 mM H_2O_2 alone (an amount far in excess of that generated in the present system) does not suppress serum EIC (data not shown). Furthermore, incubation of serum (dialyzed against 0.15 M phosphate buffer to deplete Cl^-) with PMN stimulated by PMA in a medium lacking Cl^- (160 mM sucrose, 10 mM HEPES, 0.3 mM calcium acetate and 50 mM Na phosphate, pH 7.8) also partially protected serum EIC (EIC = 70±5% of control, mean of three experiments±1 SEM). Superoxide production was not significantly affected by such treatment. The addition of Cl^- to the medium (160 mM sucrose, 10 mM HEPES, 0.3 mM calcium acetate and 50 mM NaCl, pH 7.8) eliminated protection (EIC = 49 ±8% of control, mean of three experiments±1 SEM), suggesting that inactivation of α_1-Pi is partly dependent on a halide cofactor.

In addition to the MPO system, phagocytic oxidizing capacity is believed to arise, in part, from the interaction of O_2^- and H_2O_2 (produced during the respiratory burst) with trace metal catalysts present in biological fluids (11, 23, 24). Such an interaction may give rise to more potent oxidants, thought to include species with reactivity similar to that of the hydroxyl radical (11, 23, 24). For this reason, SOD was also tested for its ability to protect serum EIC from inactivation by PMA-stimulated PMN or MNC. As shown in Table III, SOD partially protected serum EIC. SOD does not affect elastase alone (Table III). Heat-inactivated SOD failed to protect serum EIC (Table III). SOD specifically catalyzes the dismutation of O_2^- into $H_2O_2 + O_2$ (16) and was able to partially protect serum EIC. This implies that O_2^--dependent reactions may play a role in the inactivation of α_1-Pi. The observed protective effect of CAT (Table III) may be because less H_2O_2 is available for reaction with O_2^- (in addition to less H_2O_2 available to the MPO system).

Several radical scavengers, capable of reacting with ·OH but not with H_2O_2 or O_2^- (23, 25), were next tested for their ability to protect serum EIC. Mannitol (Table III), benzoate (20 mM), or ethanol (30 mM) (data not shown) failed to protect serum EIC from PMA-stimulated PMN, MNC, or PAM. In the present system this suggests that oxidants as reactive as hydroxyl radical are probably not the proximate oxidants responsible for inactivation of α_1-Pi.

Our results therefore indicate that the suppression of serum EIC by PMA-stimulated phagocytes is effected in part by the MPO-system and by O_2^--dependent reactions as well. The partially protective effect of azide or cyanide (Table III) and Cl^- depletion (see above) implicate the MPO system. In addition, the partially protective effect of SOD (Table III) suggests that O_2^--dependent reactions may also be involved. Furthermore, by combining SOD and azide, serum EIC is essentially completely protected (Table III). The complete protection provided by CAT (Table III) is also consistent with the proposed involvement of H_2O_2 in both MPO and O_2^--dependent pathways of α_1-Pi inactivation.

Further study is necessary to determine whether

TABLE III

Protection of Serum EIC from Inactivation by PMA-stimulated Phagocytes

Incubation mixture*				Assay Results‡	
Cell type	PMA (in CH_3OH)	Serum	Protective agent	O_2^-	EIC
PMN	+	+	–	100	45±6
PMN	+	+	SOD	0	64±7
PMN	+	+	CAT	95±7	89±5
PMN	+	+	HISOD	97±8	42±6
PMN	+	+	HICAT	96±8	47±5
PMN	+	+	NaN_3	95±9	75±6
PMN	+	+	KCN	103±9	73±8
PMN	+	+	Mannitol	102±8	46±7
MNC	+	+	–	100	52±8
MNC	+	+	SOD	0	70±6
MNC	+	+	CAT	97±8	95±7
MNC	+	+	HISOD	103±7	50±7
MNC	+	+	HICAT	95±9	54±8
MNC	+	+	NaN_3	101±8	77±6
MNC	+	+	Mannitol	96±8	50±9
PMN	+	+	SOD + NaN_3	0	92±6
–	+	–	SOD	—	0§
–	+	–	CAT	—	0§
–	+	–	NaN_3	—	0§

* Concentrations: SOD and heat inactivated SOD (HISOD) = 400 U/ml; CAT and HICAT = 1,500 U/ml; NaN_3 = 0.3 mM; mannitol = 30 mM; KCN = 0.5 mM. All other concentrations and conditions are as described in Table I.

‡ The results are expressed as the mean of three experiments±1 SEM. EIC as defined in Table 1.

§ Controls for the effects of all reagents on the activity of elastase. EIC = 0 represents 100% enzyme activity.

these reactions are responsible for the observed inactivation of α_1-Pi by PAM. It is currently thought that mature human macrophages do not contain MPO (20). However, macrophages may contain other forms of peroxidase (20, 26), so that these cells may also be able to catalyze peroxidative reactions.

Suppression of EIC of α_1-Pi by MPO + glucose oxidase + Cl^- in a cell-free system. The data suggesting that the MPO system is partly responsible for oxidative inactivation of α_1-Pi by PMA-stimulated PMN and MNC are consistent with our previous report (12). There we showed that an azide-inhibitable factor, possibly MPO, was also partly responsible for inactivation of α_1-Pi by PMN stimulated with opsonized immune complexes. In addition, purified MPO, in a cell-free system, has been shown to inactivate purified α_1-Pi at a pH of 6.1 (13). The pH in the vicinity of phagocytes is not known with precision. Even at sites of acute inflammation, however, extracellular pH is thought not to fall below 6.8 (27). Thus, if MPO can catalyze the inactivation of α_1-Pi extracellularly, the effect should be demonstrable at physiological pH.

We tested this possibility in vitro. Serum or purified α_1-Pi (both dialyzed against 0.05 M phosphate buffer (pH 7.4) for 6 h in the cold to remove Cl^-) were incubated with MPO plus a glucose oxidase H_2O_2-generating system and Cl^-. After 15 min at 37°C in a gyrorotary water bath, the EIC was assayed as described in Methods. As shown in Table IV, MPO + glucose oxidase + Cl^- suppressed the EIC of serum or purified α_1-Pi. MPO + glucose oxidase + Cl^- had no effect on elastase activity alone (Table IV). We determined that the presence of enzymatically active MPO was essential for suppression of EIC since NaN_3, an inhibitor of MPO activity, prevented the suppression and heat-inactivated MPO was also ineffective (see Table IV). Hydrogen peroxide (supplied by glucose oxidase) was also required, because catalase prevented suppression of serum EIC (Table IV). However, H_2O_2 alone (supplied by glucose oxidase) failed to suppress serum EIC (Table IV). In addition, the presence of a halide cofactor (Cl^-) was also essential for suppression (Table IV).

Decreased serum EIC after incubation with the MPO system was confirmed by IEP (Fig. 1D, and E). In addition, treatment of MPO-inactivated serum with DTT (under conditions identical to those previously used to

TABLE IV

Suppression of the EIC of Serum or Purified α_1-Pi by MPO + Glucose Oxidase + Cl^-

Incubation mixture*					Protective agent	Assay results‡
MPO	Glucose oxidase	Cl^-	Serum	α_1-Pi		EIC
–	–	–	+	–	–	100
+	+	+	+	–	–	13±4
–	+	+	+	–	–	97±8
+	–	+	+	–	–	97±9
+	+	–	+	–	–	101±8
HIMPO	+	+	+	–	–	102±8
+	+	+	+	–	NaN_3	95±7
+	+	+	+	–	CAT	93±10
+	+	+	+	–	HICAT	12±6
+	+	+	+	–	Mannitol	14±8
+	+	+	+	–	SOD	16±9
–	–	–	–	–	–	0§
+	+	+	–	–	–	0§
+	+	+	–	–	NaN_3	0§
+	+	+	–	–	CAT	0§
–	–	–	+	–	NaN_3	95±6
–	–	–	+	–	CAT	97±5
–	+	+	–	+	–	95±8
+	+	+	–	+	–	4±5

* Concentrations: MPO and heat-inactivated MPO (HIMPO) = 30 mU/ml; glucose oxidase = 50 mU/ml; NaCl = 0.15 M; human serum = 7.5% vol/vol (dialyzed against 0.05 M phosphate buffer, pH 7.4, before use); purified α_1-Pi = 0.15 $\mu g/\mu l$ (dialyzed against 0.05 M phosphate buffer, pH 7.4, before use); CAT or HICAT = 2,500 U/ml; NaN_3 = 2 mM; SOD = 400 U/ml; mannitol = 30 mM. The final concentration of buffer was 0.05 M phosphate, containing 30 mM glucose (pH 7.4).

‡ Results are expressed as the mean of three experiments±1 SEM. EIC as defined in Table I.

§ Controls for the effects of all reagents on the activity of elastase. EIC = 0 represents 100% enzyme activity.

reactivate phagocyte-inactivated α_1-Pi) resulted in 80±7% (mean of three experiments±1 SEM) recovery of EIC. Reactivation of α_1-Pi was also confirmed by IEP (Fig. 1F).

Experiments with metabolically-defective phagocytes. Our results suggest that O_2^-, H_2O_2, and MPO generate oxidants capable of inactivating α_1-Pi. We supported this conclusion further with an experiment using MNC and PMN from a patient with chronic granulomatous disease (CGD). Leukocytes from patients with this disorder do not undergo an effective respiratory burst during phagocytosis and do not generate significant concentrations of O_2^- or H_2O_2 (11). After incubation with PMA, neither CGD-PMN nor CGD-MNC produced detectable levels of O_2^- and they also failed to suppress serum EIC (Table I). The findings with CGD leukocytes strongly imply that inactivation of α_1-Pi depends on phagocyte products generated by the respiratory burst.

DISCUSSION

Previous work has implicated phagocyte-derived oxidants, whose generation is dependent on H_2O_2 and O_2^-, as tissue damaging agents in inflammation because of oxidant cytotoxicity (28), oxidant-mediated degradation of structural polysaccharides (29), oxidant-mediated microbicidal effects, and the reported anti-inflammatory effects of SOD (30). MPO has been shown to be responsible for tumor cell cytotoxicity (22) and platelet stimulation (31) mediated by activated PMN. In a cell-free system, MPO has also been shown to inactivate the chemotactic factors C_{5a} and f-Met-Leu-Phe (21). In addition, oxidants released from inflammatory cells at sites of acute or chronic inflammation could alter the local balance between proteases and anti-proteases. This would result from the oxidative inactivation of α_1-Pi, rendering adjacent tissue structures more susceptible to damage by proteases simultaneously released from inflammatory phagocytes. This is supported by our data as well as by previous studies (12, 13).

Hydrogen peroxide and O_2^- produced during the respiratory burst may be released extracellularly and interact to generate oxidants capable of inactivating α_1-Pi. In this context, it is important to note that normal extracellular fluids contain only traces of SOD and CAT

(29). In addition, MPO released from the lysosomal granules of PMN and MNC, H_2O_2 secreted by these cells, and extracellular halide may also catalyze the oxidative inactivation of α_1-Pi. Thus, a spectrum of oxidative mechanisms exists through which phagocytes may inactivate α_1-Pi in their local microenvironment.

Human α_1-Pi contains a methionine residue in its elastase-combining site (7, 9). Oxidation to the sulfoxide form of methionine thioether residues in or near the active site of α_1-Pi is associated with the loss of functional activity of the inhibitor (7–10) and may be the mechanism of α_1-Pi inactivation by phagocyte oxidants. In fact, MPO has been shown to oxidize two methionine residues in purified α_1-Pi, at slightly acid pH (13). Methionine oxidation has also been proposed as the mechanism for the MPO-catalyzed inactivation of the chemotactic factors C_{5a} and f-Met-Leu-Phe (21). Oxidation of thio-ether residues to the sulfoxide form is a reversible reaction (32), which is consistent with our finding that reduction can reactivate phagocyte-inactivated α_1-Pi.

In our whole-cell system, the proximate oxidant(s) responsible for inactivation of α_1-Pi appear(s) to be less reactive than ·OH since EIC was not protected by ·OH scavengers (see Table III). Although a role for ·OH must still be considered, data derived from using ·OH scavengers in intact cell systems must be interpreted with caution. This is because the degree of inhibition reflects not only rate constants of interaction between scavengers and radicals, but also the ability of the scavengers to gain access to sites of radical generation. Furthermore, serum components may react with ·OH to generate secondary radicals unreactive toward ·OH scavengers, but sufficiently reactive to inactivate α_1-Pi. Under different conditions (12), where PMN stimulated by opsonized antigen-antibody complexes were used to suppress the serum EIC, agents similar to the hydroxyl radical did appear to be involved. Oxygen metabolites generated by PMN may differ in nature and quantity, depending on the agent and conditions used to stimulate phagocyte respiration (33). This may help explain why our present data concerning the role of ·OH contrast with our earlier results (12). Further studies are necessary to identify the proximate oxidant(s) involved in α_1-Pi inactivation by stimulated phagocytes and to clarify the reactions and conditions leading to their generation.

DTT reversed the inactivation of α_1-Pi by a cell-free MPO system or by PMA-stimulated phagocytes. We do not know if reducing systems are present in vivo that are capable of reactivating oxidized α_1-Pi. Such systems could, however, play a role in modulating tissue damage at sites of inflammation and should be studied further.

Finally, it has been demonstrated recently that immunologically stimulated murine macrophages can produce more O_2^- when treated with PMA (34). Other evidence suggests that alveolar macrophages lavaged from the lungs of cigarette smokers can also produce more O_2^- (35). The increased oxidative capacity of smokers' macrophages may enhance the oxidative inactivation of α_1-Pi by macrophages in these individuals' lungs. Oxidative inactivation of α_1-Pi in smokers' lungs could render alveolar connective tissue more susceptible to the destructive action of leukocyte proteases. Such mechanisms may play a role in the pathogenesis of pulmonary emphysema in cigarette smokers. This may help explain the recent observation that uncomplexed α_1-Pi in the lungs of smokers is partly inactivated (36). It is also possible, however, that oxidants in cigarette smoke itself are responsible for this effect (8, 37).

Many questions remain, and further studies are necessary to firmly establish whether oxidative inactivation of α_1-Pi by phagocyte-derived oxidants plays a role in mediating tissue injury at sites of inflammation in vivo. However, we believe that our observations strongly indicate this possibility and render it worthy of further study.

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