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PROTEASE INHIBITORS ANTAGONIZE THE ACTIVATION OF POLYMORPHONUCLEAR LEUKOCYTE OXYGEN CONSUMPTION

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SUMMARY: We report that the burst of oxygen consumption, as well as the resultant production of $O_2^-\cdot$ and H_2O_2, occurring in activated human polymorphonuclear leukocytes is inhibited by various compounds which have in common the ability to antagonize the effects of proteolytic enzymes. This effect of protease inhibitors was observed with a variety of stimuli, both phagocytic and non-phagocytic, used to activate $O_2^-\cdot$ production in human polymorphonuclear leukocytes. Inhibition was also noted in rat polymorphonuclear leukocytes and alveolar macrophages. The results indicate that proteolysis may be involved in activating the burst of oxygen consumption following stimulation of phagocytic cells.

INTRODUCTION

Phagocytosis of bacteria by polymorphonuclear leukocytes (PMN) is accompanied by a burst of O_2 consumption that results in the formation of $O_2^-\cdot$ and H_2O_2 (1-2). A similar process occurs in other phagocytic cells (3). The importance of this event to leukocyte antibacterial activity is indicated by the frequent infections observed in children with chronic granulomatous disease whose PMN lack this burst of O_2 consumption despite normal phagocytosis and degranulation (1,4).

In addition to the phagocytosis of bacteria there are a number of other soluble and insoluble stimuli which lead to activation of the pyridine nucleotide oxidase responsible for the burst of O_2 consumption in PMN (5-6). All of these stimuli are followed by a lag period of at least 20-30 seconds. However the molecular mechanisms responsible for the activation process remain unknown.

METHODS

Materials: Cytochrome C, horse heart, Type III; superoxide dismutase, type 1, bovine blood, e-aminocaproic acid; F-Met-Leu-Phe, p-tosyl-L-arginine methyl

Abbreviation: PMN, polymorphonuclear leukocytes.

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ester and benzamidine were obtained from Sigma. Soybean and lima bean trypsin inhibitor were purchased from Worthington. Phosphoramidon, leupeptin pepstatin, chymostatin, elastatinal and antipain were obtained through the U.S.-Japan Cooperative Cancer Research Program. Phorbol myristate acetate was purchased from P. Borchert, Univ. of Minn. Trasylol and diisopropylfluorophosphate were gifts from T. Finlay. The calcium ionophore A23187 was provided by R.L. Hamill, Lilly Laboratories. Ac-Ala-Ala-Pro-Ala-CH_2Cl was a gift from J. Powers.

Cell Preparation: Blood was obtained from healthy human volunteers in accordance with the Declaration of Helsinki. After preparation by sedimentation in 1% dextran (m.wt.264,000) at 25°C for 45 min., and osmotic shock to remove red cells, PMN were resuspended in balanced salt solution (128mM NaCl, 12mM KCl, 1mM $CaCl_2$, 2mM $MgCl_2$, 2mM glucose, 4mM PO_4, pH 7.4) at a cell concentration of 10^7/ml. Rat PMN were isolated in a similar manner from the pooled aortic blood of four female Sprague Dawley rats. The lavage fluid from the lungs of these rats was pooled for the preparation of alveolar macrophages by both hypaque-ficoll gradient (7) and by a second procedure in which lavage cells adherent to plastic petri dishes were studied (8).

Assays of oxygen activation: O_2 consumption was measured as previously described (9) using equipment located in the laboratory of M.J. Broekman and A.J. Marcus. $O_2^-\cdot$ was detected by the reduction of ferricytochrome C that was inhibited by superoxide dismutase (1). In some studies, mixtures containing 1.75×10^6 cells/ml and 44 μM cytochrome C were incubated for 10 min. at 37° with activators in the absence and presence of inhibitors. After incubation, the cells were pelleted and the absorbance of the supernatant was recorded from 600-480 nm on a Perkin Elmer 552 spectrophotometer. $O_2^-\cdot$ production, as measured by cytochrome C reduction, was calculated ($E=21.1\ nM^{-1}\ cm^{-1}$ at 550 nm) using a baseline value obtained from a sample containing the activator and superoxide dismustase, 34 μg/ml. In other studies, the reduction of cytochrome C was measured at 37C using a continous assay with samples containing 0.72×10^6 cells/ml, 45 μM cytochrome C, 0.35 μg phorbol myristate acetate/ml with and without added inhibitors. Hydrogen peroxide production was assayed by following the decrease in scopoletin fluorescence (MPF-3 Perkin Elmer fluorescence spectrophotometer) as described by Root et al (2). In each study control samples contained the solvent used to solubilize the inhibitor.

RESULTS AND DISCUSSION

The addition of phorbol myristate acetate to human PMN results in a burst of O_2 consumption beginning in approximately 40 seconds (figure 1). In the presence of the protease inhibitors diisopropylfluorophosphate, soybean trypsin inhibitor, or phosphoramidon, phorbol myristate acetate produces little overt change in resting PMN oxygen consumption. Addition of phosphoramidon to PMN previously stimulated by phorbol myristate acetate results in inhibition of O_2 consumption (fig. 1). Inclusion of benzamidine (120 mM) in the PMN incubation mixture produced 78% inhibition while trasylol (80 units/ml) or epsilon-aminocaproic acid (80 mM) resulted in less than 10% inhibition of stimulated O_2 consumption.

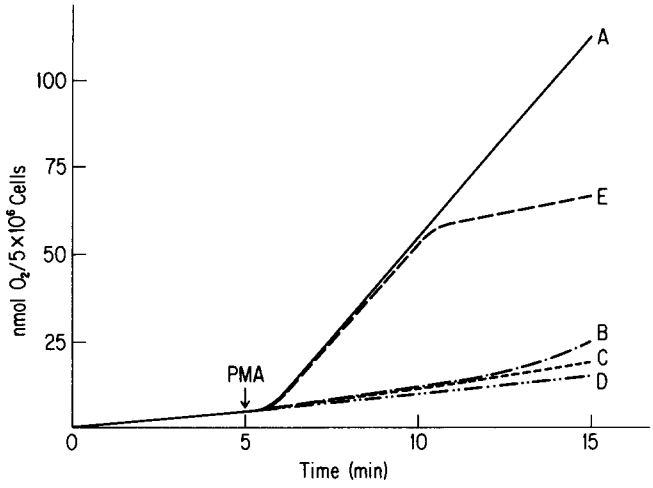

Figure 1. Oxygen consumption of stimulated polymorphonuclear leukocytes. After a 5 minute equilibration period with or without protease inhibitors phorbol myristate acetate was added to a final concentration of 118 ng/ml. Curve A, no inhibitor; curve B, 133 μM soybean trypsin inhibitor; curve C, 17 mM phosphoramidon; curve D, 2.7 mM diisopropylfluorophosphate; curve E, no inhibitor initially, 13 mM phosphoramidon added at ten minutes. PMN oxygen consumption in the absence of inhibitors was 110.4 ± 18.3 (mean ± SD) nanomoles/5 x 10^6 cells/10 min.

Further evidence of an effect of protease inhibitors was obtained in studies of PMN superoxide anion radical production, as measured by the reduction of cytochrome C (Table 1). The decrease in PMN cytochrome C reduction in the presence of protease inhibitors could be due to an interference of these compounds in the assay system, e.g. having superoxide dismutase-like effects. This was tested by adding various inhibitors in the concentrations used in the present study to a cytochrome C reducing system consisting of acetaldehyde 4.6 x 10^{-3} M, xanthine oxidase 77 μg/ml, ferricytochrome C 75 μM, and, as necessary, superoxide dismutase 34 μg/ml This mixture produced $O_2^-\cdot$ at a rate less than one tenth of that of stimulated PMN (approximately 1 nmol/min/ml). In the presence of 3mM diisopropylfluorophosphate there was a complete inhibition of cytochrome C reduction. 20mM phosphoramidon, 160 μM soybean trypsin inhibitor and 430 μM lima bean trypsin inhibitor produced 10-30% inhibition of cytochrome C reduction in this system while 135 mM benzamidine had no effect. A partial interference

Table 1. Effect of protease inhibitors on O_2^- production in polymorphonuclear leukocytes stimulated by phorbol myristate acetate.

Compound	Concentration	Percent of control
Soybean trypsin inhibitor	160μM	3
	80μM	53
	16μM	96
	1.6μM	98
Lima bean trypsin inhibitor	430μM	50
Pepstatin	750μM	99
Phosphoramidon	20mM	5
	5mM	95
Benzamidine	135mM	15
	67mM	45
	27mM	94
Epsilon-aminocaproic acid	80mM	100
Leupeptin	60mM	100
Antipain	60mM	44
	25mM	74
	3.5mM	101
TAME*	30mM	100
Trasylol	100 units/ml	99
Elastatinal	20mM	64
	40mM	37
Ac-Ala-Ala-Pro-Ala-CH_2Cl	4.8mM	89
Chymostatin	2.8mM	62

* p-Toluenesulfonyl L-arginine methyl ester

of soybean trypsin inhibitor, as well as other proteins, in PMN nitro-blue tetrazolium reduction was previously noted by Amano et al (10). Our findings indicate that only the use of diisopropylfluorophosphate would be precluded for studies depending upon cytochrome C reduction. In view of the importance of the effects of this serine esterase inhibitor toward interpreting the studies, it was elected to further confirm the inhibition of O_2 consumption by antiprotease compounds using a third measure related to activated PMN O_2 consumption, that of H_2O_2 production. A greater than 50% decrease in the rate of PMN H_2O_2 production following stimulation with 125 ng/ml phorbol

myristate acetate or 10 μg/ml digitonin was observed in the presence of 9×10^{-4} diisopropylfluorophosphate. A 40% prolongation of the lag period before H_2O_2 production was observed at a diisopropylfluorophosphate concentration of 1×10^{-4}M. The effects of other protease inhibitors on H_2O_2 production were similar to those observed in the studies of $O_2^-\cdot$ shown in Table 1.

Rat PMN and alveolar macrophage suspensions stimulated with phorbol myristate acetate produced 11.1 nmole $O_2^-\cdot$/1.2×10^6 cells/10 min and 20.7 nmole $O_2^-\cdot$/6.2×10^5 cells/30 min respectively. Soybean trypsin inhibitor, 160 μM, inhibited PMN $O_2^-\cdot$ production by 97% and that of alveolar macrophages by 100%. Similarly, when measured as described by Johnston et al (8), 160 μM soybean trypsin inhibitor resulted in complete inhibition of $O_2^-\cdot$ generation by the alveolar macrophage preparation adherent to plastic petri dishes. The ability of soybean trypsin inhibitor to inhibit PMN $O_2^-\cdot$ production stimulated by a variety of agents, both phagocytic and non-phagocytic, is shown in Table 2. These findings suggest that protease inhibitors are antagonizing a general activation process common to phagocytic cells.

The pattern of inhibitory effects does not clearly pinpoint any single PMN protease as being responsible for the activation process. The neutral proteases of human PMN include elastase, collagenase, and cathepsin G, a chymotrypsin-like enzyme, all located in intracellular granules. The inhibitory effects of diisopropylfluorophosphate implicate a serine esterase, e.g. cathepsin G or elastase. Relatively minor effects were observed with trasylol or chymostatin, both of which would be expected to inhibit cathepsin G, and a potent inhibitor of PMN elastase Ac-ala-ala-pro-ala-CH_2Cl had little effect (11). In a recent abstract (12) it has been suggested that a chymotrypsin-like enzyme is involved in the activation of human PMN $O_2^-\cdot$ production. However, this to a large extent is based on inhibition by chloromethylketone derivatives which are known to have nonspecific effects (13). Interpretation of protease inhibitor effects is complicated by lack of

Table 2. Inhibition by soybean trypsin inhibitor (SBTI) of $O_2^-\cdot$ production activated in human polymorphonuclear leukocytes by particulate and non-particulate stimuli.*

Initiator	$O_2^-\cdot$ production (nmoles/1.75 x 10^6 PMN) Control	SBTI (160 μM)
Zymosan (10 particles/cell)	9	1
Latex (10 particles/cell)**	1	0
Phorbol myristate acetate (1.72 μg/ml)	25	0
Concanavalin A (31 μg/ml)	7	0
Ionophore A23187 (0.86 μg/ml)	22	0
NaF (0.02M)	19	0
F-Met-Leu-Phe (0.6 μM)	4	1
Digitonin (10 μg/ml)	5	0

* Samples were incubated at 37°C for 10 minutes except for the NaF study in which the incubation time was 20 minutes.
** Sample contained 0.172 ml serum/ml buffer. Particles 1.099 microns in diameter.

information concerning the location of the added inhibitor in relation to the unknown site of activation. This need not be in PMN granules as following stimulation the granules fuse with the cell membrane, where $O_2^-\cdot$ is produced (14). Other PMN proteases have also been reported, including a cell membrane enzyme inhibitable by diisopropylfluorophosphate and soybean trypsin inhibitor (15). It is also conceivable that there is a cascade of events during the lag period involving more than one protease, as well as changes in membrane polarity (16) and fluidity, leading to pyridine nucleotide oxidase activation. A direct effect of protease inhibitors on this latter enzyme can not be completely precluded, particularly in view of the reported similarity in active site configuration between proteases and other enzyme types (17).

Degranulation and enzyme release in PMN of chronic granulomatous disease patients is thought to be normal. However, the literature reveals

no instances in which neutral proteases were measured. It would be of interest to do so, as well as to test for the presence of a protease inhibitor.

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