SERINE PROTEASE INHIBITORS INHIBIT SUPEROXIDE PRODUCTION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND MONOCYTES STIMULATED BY VARIOUS SURFACE ACTIVE AGENTS

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1. Introduction

We have recently shown that chymotrypsin-like serine proteases, which exist in an enzymatically active form on the cell surface membrane, are involved in the superoxide ($O_2^{\cdot-}$) production by human polymorphonuclear leukocytes (PMN) and monocytes stimulated by cytochalasin E and concanavalin A [1,2]. To clarify the role of the serine proteases, we investigate here whether the same serine proteases are also involved in the $O_2^{\cdot-}$ production induced by other soluble agents, including wheat germ agglutinin (a lectin), *N*-formylmethionyl phenylalanine (a chemotactic peptide), phorbol myristate acetate (an active principle of croton oil), calcium ionophore A23187 and phospholipase C, which appear to initiate the oxidative metabolism of phagocytic cells through different mechanisms [3–11]. The $O_2^{\cdot-}$ production by human PMN and monocytes stimulated by these active agents was also inhibited by irreversible serine protease inhibitors and synthetic substrates for serine proteases, indicating that the serine proteases are essential for human PMN and monocytes to release $O_2^{\cdot-}$.

Abbreviations: PMN, polymorphonuclear leukocytes; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid; SOD, superoxide dismutase; FMP, *N*-formylmethionyl phenylalanine; PMA, phorbol myristate acetate; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonylfluoride; TPCK, L-1-tosylamido-2-phenylethyl-chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; TAME, *p*-tosyl-L-arginine methyl ester

2. Materials and methods

Cytochrome *c* type VI, superoxide dismutase, *N*-formylmethionyl phenylalanine, phorbol myristate acetate, phospholipase C, phenylmethylsulfonyl-fluoride, L-1-tosylamido-2-phenylethyl-chloromethyl ketone, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, *N*-benzoyl-L-tyrosine ethyl ester and *p*-tosyl-L-arginine methyl ester were purchased from Sigma Chemical Co., St Louis, MO; wheat germ agglutinin (WGA) from EY Labs., San Mateo, CA; calcium ionophore A23187 was a gift from Dr R. J. Hosley, Eli Lilly Co., Indianapolis, IN. PMSF, TPCK, BTEE, FMP, PMA and A23187 were dissolved in dimethylsulfoxide and diluted with Hepes–saline (isotonic saline solution buffered with 5 mM *N*-2-hydroxy-ethyl-piperazine-*N'*-2-ethane sulfonic acid, pH 7.4) immediately before use. The final concentration of DMSO in the reaction mixture was < 5 μl/ml and the same concentration of DMSO was added in the controls.

2.1. *Preparation of cells*

PMN and mononuclear cell preparations were obtained from healthy adult donors by the dextran sedimentation and Conray-Ficoll method as in [1,2]. Both cell preparations were suspended in Hepes–saline. PMN preparations contained $> 99\%$ PMN. Mononuclear cell fractions were used as monocyte fractions, which contained 15–25% monocytes and $< 1\%$ PMN by morphological criteria. The rest was composed of lymphocytes. Monocytes are considered to be responsible for the $O_2^{\cdot-}$ production by mono-

nuclear cells, since the $O_2^{\cdot-}$ production by non-phagocytic cells (> 99% lymphocytes) is negligible when phagocytic cells ingesting carbonyl iron particles are eliminated by a magnet from the mononuclear cell fractions [2].

2.2. *Determination of PMN and monocyte $O_2^{\cdot-}$ production*

$O_2^{\cdot-}$ was assayed by SOD-inhibitable cytochrome *c* reduction spectrophotometrically, using a Hitachi 557 spectrophotometer (a double wavelength spectrophotometer with end-on photomultiplier) as in [1]. The cell suspension was added to a 1 ml cuvette containing 2 mM glucose, 1 mM $CaCl_2$ and 66 μM ferricytochrome *c* with or without test materials to obtain 0.99–0.995 ml final vol. The reaction mixture in the cuvette was preincubated at 37°C for 3 min for protease inhibitors and for 10 min for synthetic substrates of serine proteases, respectively. The cuvette was put into the thermostated cuvette holder (37°C) of a spectrophotometer and the reduction of cytochrome *c* was measured at 550 nm with a reference wavelength at 540 nm. Various stimulating agents (5–10 μl) were added to the reaction mixtures in cuvettes to obtain 1 ml final vol. and the desired concentrations of these agents. Final cell concentrations were variable in accordance with the potencies of the active agents. In PMN $O_2^{\cdot-}$ production studies, 4–8 × 10^5/ml were used for PMA; 1–3 × 10^6/ml for WGA, FMP and A23187; and 1 × 10^7/ml for phospholipase C, respectively. In monocyte $O_2^{\cdot-}$ production studies, 1–3 × 10^6 mononuclear cells/ml were used for WGA, PMA and FMP. In these studies, cell viability by the erythrosine B dye-exclusion test was always checked after the assay of $O_2^{\cdot-}$ production and was > 95%.

3. Results and discussion

WGA, FMP, PMA, A23187 and phospholipase C were able to stimulate human PMN and monocytes to release $O_2^{\cdot-}$ (fig.1) [3–11], however A23187 and phospholipase C were toxic for mononuclear cells but not for PMN at the concentrations used in the present experiments. Therefore, A23187 and phospholipase C were excluded from the monocyte $O_2^{\cdot-}$ production studies. The representative time courses of PMN $O_2^{\cdot-}$ production are shown in fig.1. The almost similar patterns were observed in monocyte

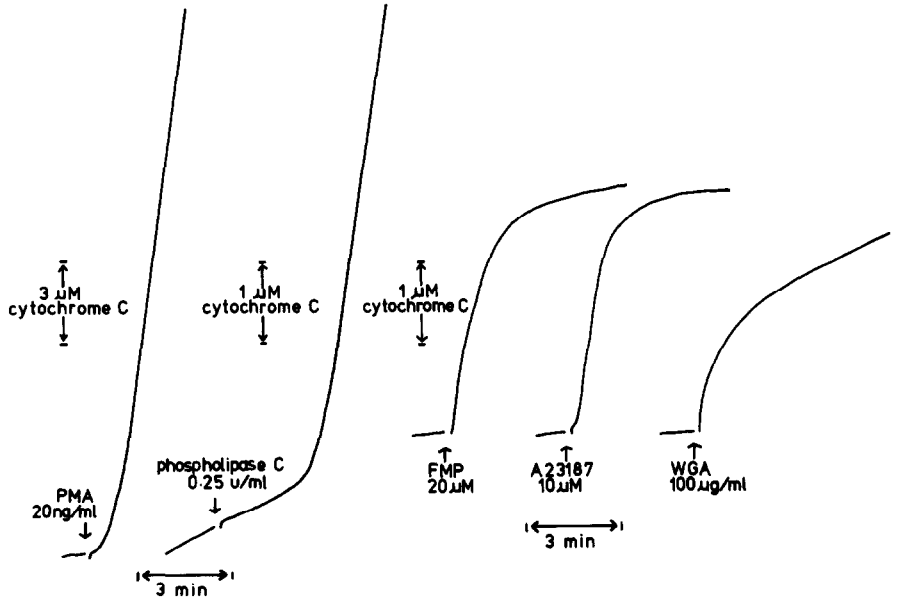

Fig.1. $O_2^{\cdot-}$ production by human PMN stimulated by various active agents. Cell concentrations used were 8 × 10^5/ml for PMA, 1 × 10^7/ml for phospholipase C and 2 × 10^6/ml for FMP, A23187 and WGA.

$O_2^{\cdot-}$ production (data not shown). Cytochrome *c* reduction induced by these active agents was completely abolished by SOD (20 μg/ml) suggesting it to be specific for $O_2^{\cdot-}$. The $O_2^{\cdot-}$ production by human PMN and monocytes was calculated from an apparently linear portion in PMA and phospholipase C induced $O_2^{\cdot-}$ production [1], and was calculated from cytochrome *c* reduced for 2 min after the addition of FMP or A23187 and for 5 min after the addition of WGA (fig.1). The values of cytochrome *c* reduced in the resting states were subtracted from those in the stimulated states.

As shown in table 1, irreversible serine protease inhibitors (PMSF, TPCK and TLCK) and synthetic substrates for serine proteases (BTEE and TAME) inhibited the $O_2^{\cdot-}$ production by human PMN and monocytes induced by several active agents, including WGA, FMP, PMA, A23187 and phospholipase C, the actions of which are suggested to be different from one another. WGA binds to (β(1→4)-D-*N*-acetylglucosamine)$_2$ on the cell surface membrane [12]. FMP and PMA are also suggested to bind to the cell surface membrane [13,14], although it is uncertain what molecules are the binding sites. It is probable that membrane perturbation provoked by the binding of these active agents on the cell surface membrane is responsible for the $O_2^{\cdot-}$ production. On the other hand, phospholipase C acts on the membrane lipid and hydrolyzes phosphatidylcholine [11], which is a major component of the outer layer of the cell membrane. It is unknown how phospholipase C is able to provoke the oxidative metabolism, while it is possible that the hydrolysis of phosphatidylcholine results in the conformational changes of the membrane and allows the redistribution of the macromolecules on the surface membrane, which in turn activates the NAD(P)H oxidase. A23187 is suggested to initiate the oxidative metabolism by promoting Ca^{2+} influx from the milieu [8]. It has been reported that extracellular Ca^{2+} plays an important role and enhances the oxidative metabolism induced by *N*-formylmethionyl peptides and phos-

Table 1
Inhibitory effect of serine protease inhibitors and synthetic substrates for serine proteases on PMN and monocyte $O_2^{\cdot-}$ production induced by various surface active agents

	PMN $O_2^{\cdot-}$ production (% control)[a]				
	WGA (100 μg/ml)	FMP (20 μM)	PMA (20 ng/ml)	A23187 (10 μM)	phospholipase C (0.25 units/ml)
PMSF (1 mM)	34.5 ± 7.3	23.2 ± 5.5	82.6 ± 2.1	14.5 ± 2.8	n.d.
TPCK (10 μM)	27.0 ± 1.4	31.2 ± 6.3	50.9 ± 1.4	22.5 ± 8.7	11.1 ± 3.2
TLCK (100 μM)	49.1 ± 5.8	46.7 ± 1.6	74.4 ± 1.4	64.4 ± 4.0	20.4 ± 2.6
BTEE (50 μM)	n.d.	27.0 ± 4.2	77.8 ± 4.5	n.d.	n.d.
TAME (1 mM)	n.d.	79.8 ± 9.0	82.7 ± 2.8	n.d.	n.d.

	Monocyte $O_2^{\cdot-}$ production (% control)[a]		
	WGA (100 μg/ml)	FMP (20 μM)	PMA (20 ng/ml)
PMSF (1 mM)	50.8 ± 3.9	54.7 ± 6.4	68.5 ± 5.4
TPCK (10 μM)	27.3 ± 2.9	67.6 ± 1.5	52.4 ± 8.8
TLCK (100 μM)	60.4 ± 3.6	63.9 ± 6.6	65.1 ± 3.3
BTEE (50 μM)	n.d.	23.2 ± 5.6	69.3 ± 7.4
TAME (1 mM)	n.d.	69.1 ± 3.3	88.6 ± 7.9

[a] Mean ± SD. Average of 2 or 3 experiments; each experiment was done in duplicate

n.d., not done

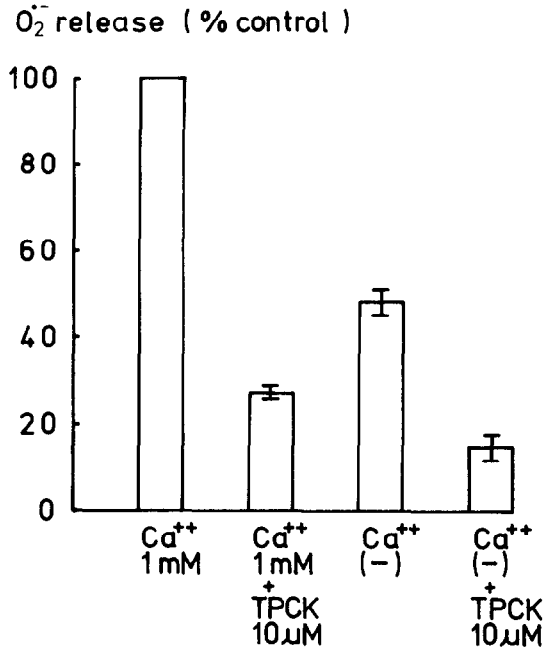

Fig.2. Effect of extracellular Ca^{++} and TPCK on PMN $O_2^{\cdot-}$ production induced by WGA. WGA (100 μg/ml) was added to the reaction mixture containing 1 mM Ca^{2+} or no Ca^{2+} with or without TPCK (10 μM). The value of $O_2^{\cdot-}$ produced in the condition containing 1 mM Ca^{2+} without TPCK is shown as 100% control. Bars denote mean ± SD.

pholipase C [3–5,11], while PMA-induced $O_2^{\cdot-}$ production is never affected by extracellular Ca^{2+} [5]. As shown in fig.2, WGA-induced $O_2^{\cdot-}$ production by human PMN was also enhanced by extracellular Ca^{2+}, and it was inhibited by TPCK whether Ca^{2+} was present in the milieu or not. These above findings indicate that the serine proteases may not be implicated in Ca^{2+} influx. Differences of the inhibition were seen when different stimulants were used (table 1). This may be partly explained by the differences of the actions among the stimulants.

The present experiments have shown that serine protease inhibitors and synthetic substrates for serine proteases are able to inhibit the $O_2^{\cdot-}$ production by human PMN and monocytes induced by various surface active agents, indicating that the serine proteases play an essential role for the $O_2^{\cdot-}$ production by human PMN and monocytes.

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