POSSIBLE INVOLVEMENT OF PROTEASES IN SUPEROXIDE PRODUCTION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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1. Introduction

Superoxide ($O_2^{\cdot-}$) production by phagocytic cells requires intact, viable cells and proceeds independently of phagocytosis. It is probably initiated at the cell surface by the interaction of cell surface membrane and appropriate stimuli [1–3]. In this paper, we investigate the effect of various protease inhibitors and synthetic substrates for serine proteases on the $O_2^{\cdot-}$ production by human polymorphonuclear leukocytes (PMN). From the inhibitory effect of these substances, it was suggested that the chymotrypsin-like serine proteases may be involved in the $O_2^{\cdot-}$ production by human PMN and that they may be located at the cell surface membrane.

2. Materials and methods

Cytochalasin E (Cyt E) was obtained from Aldrich Chemical Co.; concanavalin A (Con A) grade IV, cytochrome *c* type VI, superoxide dismutase, xanthine, xanthine oxidase grade III, L-1-tosyl-amido-2-phenylethyl-chloromethyl ketone (TPCK), *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK), soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl-fluoride (PMSF), *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and *p*-tosyl-L-arginine methyl ester (TAME) from Sigma Chemical Co.; aprotinin from Bayer Co. Cyt E, TPCK, BTEE and PMSF were dissolved in dimethylsulfoxide (DMSO) and diluted 1:1 with HEPES–saline (isotonic saline solution buffered with 5 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid, pH 7.4) immediately before use. The final concentration of DMSO in the reaction mixture was 2.5–5 μl/ml and the same volume of DMSO was added to the controls.

2.1. *Preparation of cells*

Heparinized venous blood from healthy adults donors was allowed to sediment at room temperature for 30 min after mixing with the same volume of 3% dextran in isotonic saline. The pure PMN fraction was obtained from the leukocyte rich fraction by the Conray-Ficoll method [4]. Contaminated red cells in PMN fraction were removed by hypotonic lysis. PMN fraction was suspended in HEPES–saline and contained >95% PMN.

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

$O_2^{\cdot-}$ was assayed by the reduction of ferricytochrome *c* spectrophotometrically, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double wavelength spectrophotometer with end-on photomultiplier which contains microcomputer in it), equipped with thermostatted cuvette holder [2,3]. The cell suspension was added to a 1 ml cuvette containing 2 mM glucose, 66 μM ferricytochrome *c* and 1 mM $CaCl_2$ final vol. 0.99 ml with or without test materials. Final cell concentration was $4–8 \times 10^5$/ml. The reaction mixture in a 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 in a thermostatted cuvette holder (37°C) of a spectrophotometer and the reduction of

cytochrome *c* measured at 550 nm with a reference wavelength at 540 nm, with the full scale of the recorder set at *A* 0.1 absorbance. Cyt E (5 μl, final conc. 5 μg/ml) and Con A (5 μl, final conc. 50 μg/ml) were added to the reaction mixture in a cuvette simultaneously using a plastic rod, while the time course of cytochrome *c* reduction was followed on the recorder. Cytochrome *c* reduction by human PMN stimulated by Cyt E and Con A was completely abolished by superoxide dismutase (10 μg/ml) and suggested it to be specific for $O_2^{\cdot-}$ as in [2,3]. Results from 2 or 3 experiments were averaged and converted to nmol cytochrome *c* reduced by using $E_{550-540}$ (ferrocytochrome *c* minus ferricytochrome *c*) = 15.5×10^3 M^{-1} cm^{-1} [2]. Although the cytochrome *c* reduction was not actually linear with time, we used for the assay an apparently linear portion. In these studies, cell viability by Erythrosine B dye-exclusion test was >95%.

3. Results and discussion

Cell suspensions in cuvettes were preincubated with various concentrations of protease inhibitors for 3 min at 37°C, before Cyt E (5 μg/ml) and Con A (50 μg/ml) were added. $O_2^{\cdot-}$ production by PMN was impaired by various protease inhibitors, including the active-site serine sulfonylating agent PMSF; active-site histidine alkylating agents TPCK and TLCK; and naturally occurring macromolecular inhibitors aprotinin and SBTI (fig.1). Typical results obtained with TPCK are shown in fig.2A. The inhibitory effect of TPCK was instantaneous and dose dependent even when TPCK was added to the reaction mixture at the time of maximum $O_2^{\cdot-}$ production (fig.2B). The almost similar patterns of the inhibitory effect were observed when the other protease inhibitors were used. The relative potencies of inhibitory effect were TPCK > aprotinin > TLCK > SBTI > PMSF on a molar basis (fig.1). TPCK, which inhibits chymotrypsin, was most effective; TLCK and SBTI, which inhibit trypsin, were less effective; and aprotinin, which inhibits both chymotrypsin and trypsin, was intermediate. These results indicate that chymotrypsin-like serine proteases may be involved in the $O_2^{\cdot-}$ production by human PMN. Macromolecular inhibitors (aprotinin and SBTI) also inhibited $O_2^{\cdot-}$ production by human PMN. This finding, together with the immediate inhibition observed, suggests that proteases involved in $O_2^{\cdot-}$ production by human PMN may be on the outer

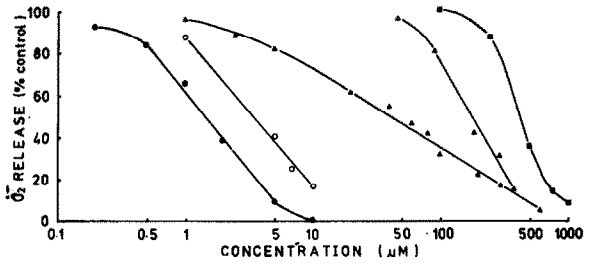
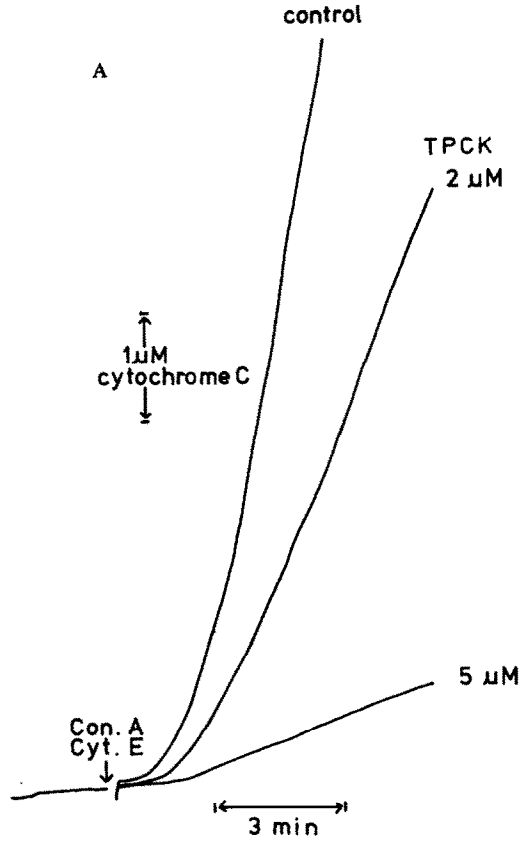


Fig.1. The inhibitory effect of various protease inhibitors on the $O_2^{\cdot-}$ production by human PMN stimulated by Cyt E (5 μg/ml) and Con A (50 μg/ml). Cell suspensions were preincubated with protease inhibitors for 3 min at 37°C before stimulators were added. (●——●) TPCK; (○——○) aprotinin; (▲——▲) TLCK; (△——△) SBTI; (■——■) PMSF.



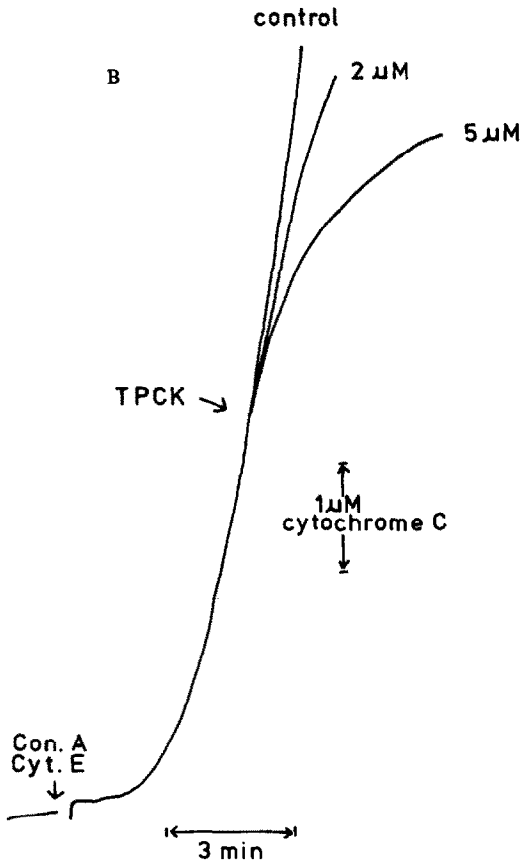


Fig.2. The inhibitory effect of TPCK on $O_2^{\cdot-}$ production by human PMN. $O_2^{\cdot-}$ production by PMN (8×10^5/ml) was induced by Cyt E (5 μg/ml) and Con A (50 μg/ml). (A) Cell suspensions were preincubated with TPCK for 3 min at 37°C before stimulators were added. (B) TPCK was added at the time of maximum $O_2^{\cdot-}$ production.

surface of PMN plasma membranes, which are accessible to these macromolecular inhibitors.

To obtain further support for the hypothesis that proteases are involved in $O_2^{\cdot-}$ production, we investigated the effect of synthetic substrates for serine proteases on the $O_2^{\cdot-}$ production. These substrates included BTEE (substrate for chymotrypsin type protease) and TAME (substrate for trypsin type protease). As shown in fig.3, the inhibitory effect of BTEE and TAME was dose dependent, and BTEE was much more effective than TAME. These results also suggest that proteases involved in $O_2^{\cdot-}$ production by human PMN may be chymotrypsin-like serine proteases, and that the synthetic substrates for serine proteases inhibit $O_2^{\cdot-}$ production by competing with natural substrates for proteases that are involved in $O_2^{\cdot-}$ production by human PMN.

The $O_2^{\cdot-}$ production by the xanthine–xanthine

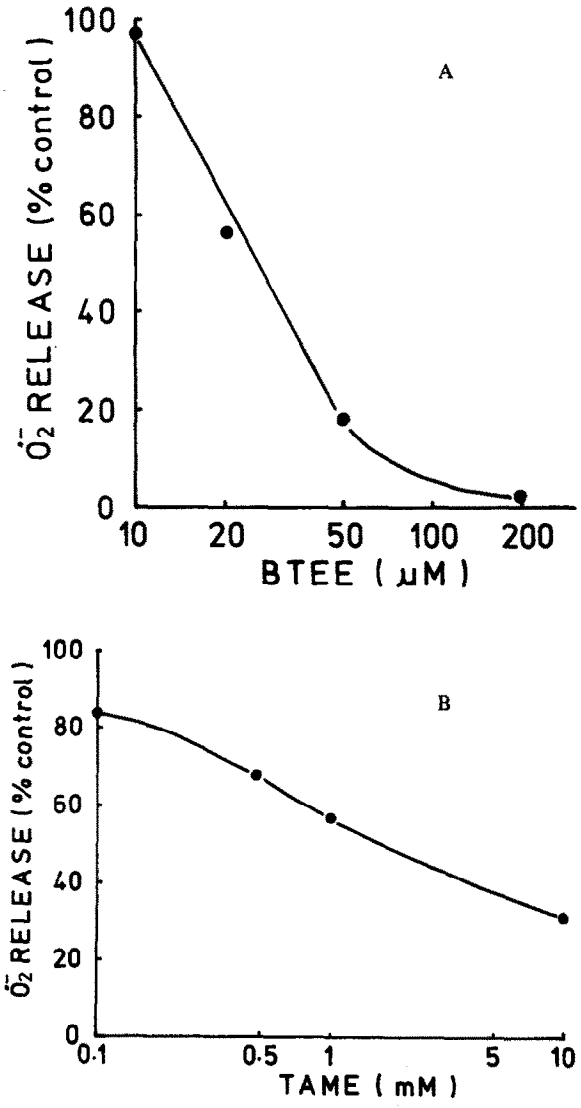


Fig.3. The inhibitory effect of synthetic substrates for serine proteases, BTEE (A) and TAME (B), on $O_2^{\cdot-}$ production by human PMN stimulated by Cyt E (5 μg/ml) and Con A (50 μg/ml). Cell suspensions were preincubated with various concentrations of BTEE or TAME for 10 min at 37°C before stimulators were added.

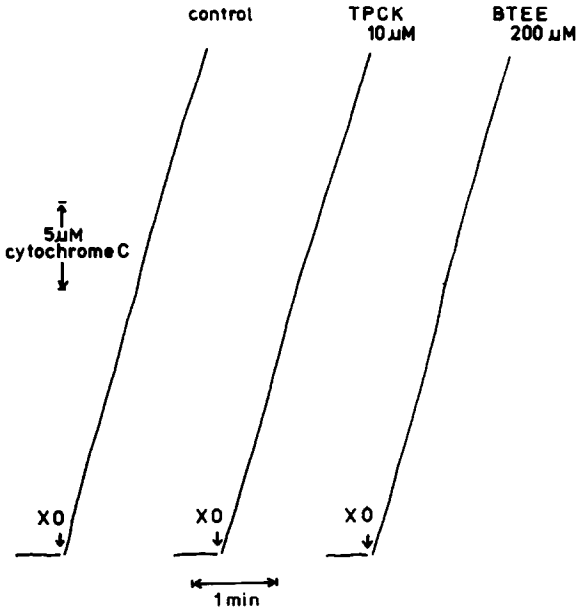


Fig.4. The effect of TPCK and BTEE on $O_2^{\cdot-}$ production by the xanthine–xanthine oxidase system. Xanthine oxidase (XO, 5 μl, final conc. 0.02 units/ml) was added to the reaction mixture (0.995 ml) containing 0.4 mM xanthine and 66 μM ferricytochrome *c* with or without test materials in HEPES–saline (pH 7.4). The reduction of cytochrome *c* was measured at 550–540 nm in a Hitachi 557 spectrophotometer at 37°C. Cytochrome *c* reduction by the xanthine–xanthine oxidase system was completely abolished by superoxide dismutase.

oxidase system [5] was not impaired by TPCK (10 μM), aprotinin (10 μM), TLCK (500 μM), SBTI (400 μM), PMSF (1 mM), BTEE (200 μM) and TAME (1 mM) as shown in fig.4 (data not shown except for TPCK and BTEE). These findings indicate that these protease inhibitors and synthetic substrates do not themselves react with $O_2^{\cdot-}$.

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