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SERINE PROTEASE INHIBITORS INHIBIT SUPEROXIDE PRODUCTION BY HUMAN BASOPHILS STIMULATED BY ANTI-IgE

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SUMMARY: Anti-IgE-induced O_2^- production by human basophils was inhibited by potent inactivators of serine proteases. The inhibitory effect of the inhibitor and substrate for chymotrypsin-type protease was much greater than that of those substances for trypsin-type protease. These findings suggest that chymotrypsin-like serine proteases are involved in basophil O_2^- production.

INTRODUCTION

It has been shown by us and other investigators that serine protease inhibitors and synthetic substrates for serine proteases inhibit superoxide (O_2^-) production by human neutrophils and monocytes stimulated by various surface active agents (1-6). In this paper, we extended the study to human leukemic basophils, which also release O_2^- in response to anti-IgE (7).

MATERIALS AND METHODS

Reagents. Cytochrome C type VI, superoxide dismutase, phenylmethylsulfonylfluoride (PMSF), L-1-tosylamido-2-phenylethyl-chloromethyl ketone (TPCK), N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK), N-benzoyl-L-tyrosine ethyl ester (BTEE), p-tosyl-L-arginine methyl ester (TAME) and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemical Co., St. Louis, Mo.; rabbit anti-human IgE from Behring Institute, West Germany. PMSF, TPCK and BTEE were dissolved in dimethylsulfoxide and diluted with HEPES-saline (isotonic saline solution buffered with 5 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, pH 7.4) immediately before use. The final concentration of dimethylsulfoxide in the reaction mixture was < 2.5 μl/ml and the same concentration of dimethylsulfoxide was added to the controls when required.

Preparation of cells. It is difficult to obtain normal human basophils sufficiently to investigate their O_2^- producing mechanism. Therefore, we used leukemic basophils in the present experiments. Human basophils were obtained from two patients with basophilia. A patient (K.T.,30-year-old Japanese female) with basophilic leukemia had a peripheral leukocyte count ranging from 80,000 to 130,000/mm^3 with 60-70% mature basophils, and another patient (F.A., 54-year-

Abbreviations: BTEE, N-benzoyl-L-tyrosine ethyl ester; PMSF, phenylmethylsulfonylfluoride; SBTI, soybean trypsin inhibitor; TAME, p-tosyl-L-arginine methyl ester; TLCK, N-α-p-tosyl-L-lysine-chloromethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl-chloromethyl ketone.

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old Japanese female) with chronic myelogenous leukemia (Philadelphia chromosome positive) had a leukocyte count ranging from 400,000 to 500,000/mm^3 with 10-20% mature basophils. The basophil preparations were obtained from heparinized venous blood with dextran sedimentation and hypotonic lysis of the remaining erythrocytes as previously described (1-3). The basophil preparations were suspended in HEPES-saline and contained 60-70% basophils for the patient K.T. and 10-20% for the patient F.A. Further purification of basophils by Conray-Ficoll method was not performed, because the leukemic basophils were redistributed by this maneuver not only to the interface layer but also to the bottom and into the Conray-Ficoll layer. Normal neutrophils and mononuclear cells were obtained from healthy adult donors with dextran sedimentation and Conray-Ficoll method as previously described (3). These cell preparations were suspended in HEPES-saline. The mononuclear cell preparations contained 15-25% monocytes, 75-85% lymphocytes and not more than 1% basophils. The neutrophil preparations contained 97-99% neutrophils and 1-3% eosinophils.

Determination of basophil O_2^- production. O_2^- was assayed by superoxide dismutase inhibitable cytochrome C reduction spectrophotometrically, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a dual wavelength spectrophotometer; Hitachi Ltd., Tokyo) as previously described (3). 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 final volume of 0.99 ml. Final cell concentration was 5-8x10^5 basophils/ml. The reaction mixture in a cuvette was preincubated at 37°C for indicated times. The cuvette was put in a thermostatted 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. Anti-IgE (10 μl) was added to the reaction mixture in a cuvette to obtain final volume of 1 ml, while the time course of cytochrome C reduction was followed on the recorder. Basophil O_2^- production was calculated from cytochrome C reduced for 5 min after the addition of anti-IgE. And the values of cytochrome C reduced in the resting states were subtracted from those in the stimulated states (Fig. 1). In these studies, cell viability by erythrosine B dye exclusion test was always checked after the assay of O_2^- production, and was $>$95% even after the treatment with various compounds.

RESULTS AND DISCUSSION

When anti-IgE (10 μl/ml) was added to the reaction mixture containing human basophils, the increase of cytochrome C reduction, which was completely abolished by superoxide dismutase (20 μg/ml), was noted to begin after a lag period of approximately 20 sec (Fig. 1) (7). On the other hand, when anti-IgE (10 μl/ml) was added to the reaction mixture containing human neutrophils or mononuclear cells (1-5x10^6 cells/ml) obtained from healthy adult donors, the increase of cytochrome C reduction was negligible (data not shown), indicating that basophils may be responsible for anti-IgE-induced O_2^- production. Because the basophil O_2^- production studies were done with heterogenous cell preparations containing neutrophils and monocytes, further control experiment was performed to exclude the possibility that lysosomal enzymes released from

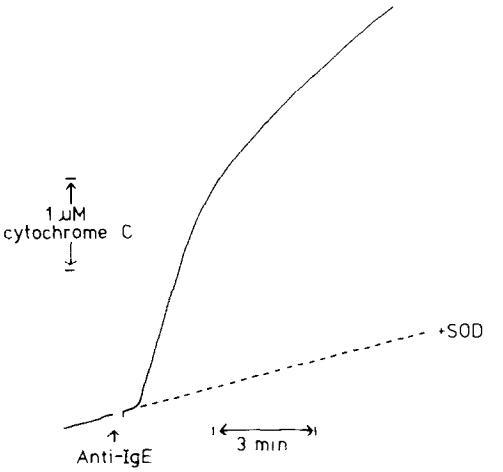

Fig. 1. Anti-IgE-induced O_2^- production by human basophils. Anti-IgE (10 μl/ml) was added to the reaction mixture containing human basophils ($5x10^5$ basophils/ml). The increase of cytochrome C reduction was completely abolished by superoxide dismutase (SOD; 20 μg/ml).

activated basophils stimulate neutrophils or monocytes to release O_2^-. Basophil preparation was incubated with anti-IgE (10 μl/ml) for 10 min at 37°C, centrifuged and the supernatant was removed. The supernatant failed to induce O_2^- production by normal neutrophils or mononuclear cells. These observations further indicate that anti-IgE-induced O_2^- production may be attributed to basophils. O_2^- production was dependent on the concentration of anti-IgE (Fig. 2) and the number of cells (data not shown).

As shown in Table I, anti-IgE-induced O_2^- production by human basophils, which were obtained from two patients with basophilia, was inhibited by potent inactivators of serine proteases (esterases), including the active-site serine sulfonylating agent PMSF (8); active-site histidine alkylating agents TPCK and TLCK (9-11); naturally occurring macromolecular inhibitor SBTI (12); and synthetic substrate for chymotrypsin-type protease BTEE (13). No significant inhibition was observed by 1 mM TAME (synthetic substrate for trypsin-type protease). TPCK and TLCK are specific inhibitors of chymotrypsin- (9) and trypsin-like (10) enzymes, respectively. The inhibition by TPCK and TLCK was dose and time dependent (Table I and Fig. 3), and their effect was irreversible (Fig. 4), an observation consistent with their proposed mechanism that they

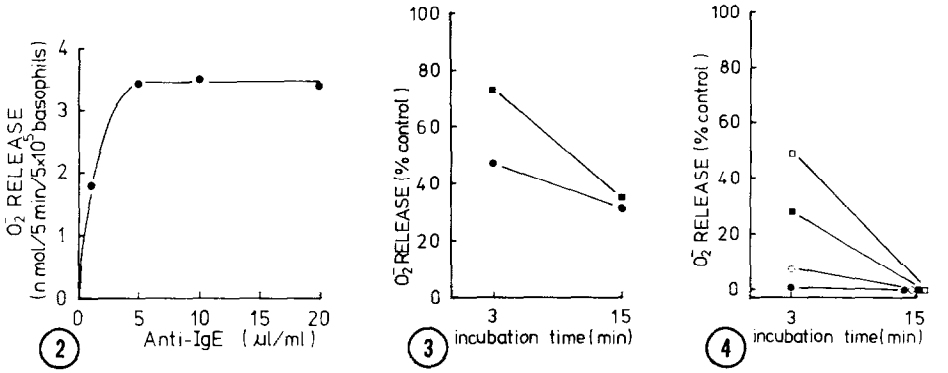

Fig. 2. Effect of the concentration of anti-IgE on the O_2^- production by human basophils.

Fig. 3. Effect of preincubation time with TPCK or TLCK on basophil O_2^- production. Cell suspensions were preincubated with TPCK or TLCK for 3 or 15 min at 37°C before anti-IgE (10 μl/ml) was added. ● , 5 μM TPCK; ■ , 100 μM TLCK.

Fig. 4. Effect of cell washing. Solid symbols indicate O_2^- production by human basophils that were preincubated with the inhibitors for 3 and 15 min at 37°C and were not washed. Open symbols indicate O_2^- production by human basophils that were preincubated with the inhibitors for 3 and 15 min at 37°C and were washed twice to remove the inhibitors from the milieu. Control cells were preincubated with the same concentrations of dimethylsulfoxide and simultaneously run. O_2^- production was induced by anti-IgE (10 μl/ml). ● , ○ , 50 μM TPCK; ■ , □ , 500 μM TLCK.

Table I

Inhibitory effect of serine protease inhibitors and synthetic substrates for serine proteases on basophil O_2^- production

	O_2^- production (% control)*	
	patient 1 (F.A.)	patient 2 (K.T.)
PMSF (1 mM)	41.6 ± 1.3	20.8 ± 3.1
TPCK (5 μM)	n.d.	47.2 ± 4.1
(10 μM)	30.5 ± 1.8	33.0 ± 0.1
(50 μM)	0.8 ± 0.1	n.d.
TLCK (100 μM)	77.6 ± 2.1	73.0 ± 4.7
(500 μM)	17.2 ± 3.6	28.1 ± 0.6
SBTI (400 μM)	65.7 ± 5.9	n.d.
BTEE (50 μM)	52.0 ± 1.6	38.1 ± 2.1
TAME (1 mM)	96.5 ± 2.3	108.4 ± 8.1

* Cell suspensions in cuvettes were preincubated with protease inhibitors (PMSF, TPCK, TLCK and SBTI) for 3 min or with synthetic substrates (BTEE and TAME) for 10 min at 37°C before anti-IgE (10 μl/ml) was added. Each value is the mean ± SD of duplicate determinations of O_2^- production (% control) in one or two experiments. Results from two different individuals are shown.

n.d. not done.

ultimately form covalent bonds at the active sites of the enzymes (9-11). The slight restoration by the washing procedure might be explained by a reversible complex as an intermediate (3,11). The inhibitory effect of the inhibitor and substrate for chymotrypsin-type protease (TPCK and BTEE) was much greater than that of those substances for trypsin-type protease (TLCK and TAME), suggesting that chymotrypsin-like serine proteases are involved in basophil O_2^- production. Chloromethyl ketone derivatives of amino acids such as TPCK and TLCK also possess a high reactivity with sulfhydryl groups and can inhibit thiol-dependent enzymes such as ficin, papain or cathepsin B_1 (14-16). Therefore, the present results may indicate that chymotrypsin-like proteases and/or thiol-dependent enzymes are required for basophil O_2^- production. However, the fact that basophil O_2^- production was also inhibited by an amino acid ester (BTEE) and other serine protease inhibitors (SBTI and PMSF) may strengthen the hypothesis that serine proteases are involved in basophil O_2^- production as has previously been inferred for O_2^- production by human neutrophils and monocytes (1-6).

A requirement of intact serine protease (esterase) activity has been suggested for antigen-induced histamine release from human basophils, since histamine release is inhibited by diisopropylfluorophosphate, an active-site serine phosphorylating agent (17,18). The present studies demonstrate a similar requirement in anti-IgE-induced O_2^- production by human basophils.

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