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# INHIBITION OF NEUTROPHIL SULFHYDRYL GROUPS BY CHLOROMETHYL KETONES

## A MECHANISM FOR THEIR INHIBITION OF SUPEROXIDE PRODUCTION

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The inhibition of $O_2^-$ production by serine protease inhibitors such as chloromethyl ketone derivatives, has been used as evidence to indicate that protease activity is essential for the production of $O_2^-$ by neutrophils. However, chloromethyl ketones are potent inhibitors of sulfhydryl groups. This study demonstrates that chloromethyl ketones inhibited non-protein sulfhydryl groups as well as $O_2^-$ production by human neutrophils stimulated with phorbol myristate acetate (PMA). Their inhibition of $O_2^-$ production could be prevented by reduced glutathione. The results suggest that inhibition of $O_2^-$ production by chloromethyl ketones is largely due to their inhibition of sulfhydryl groups.

Recently, there has been considerable interest in the role of serine proteases (or esterases) on neutrophil functions. Using potent inhibitors or synthetic substrates of serine esterases, several studies have shown that protease activity may be essential for neutrophil chemotaxis (1,2), immune erythrophagocytosis (3,4), lysosomal enzyme release (5-7), and the initiation and maintenance of superoxide ($O_2^-$) production (7-11). However, practically nothing is known about the mechanism by which the protease activity is involved in these diverse neutrophil functions, nor do we know much about the nature and localization of the enzyme. In addition, the involvement of protease activity in these neutrophil functions is primarily inferred from studies using inhibitors of serine esterases or their synthetic substrates. These inhibitors or substrates have non-specific effects (12-14). Thus, the observed effects could be due to something other than their reactions with serine esterases.

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The chloromethyl ketone derivatives of α-N-tosyl-L-phenylalanine (TPCK) and α-N-tosyl-L-lysine (TLCK) react with one of the histidines in the active sites of chymotrypsin (15,16) and trypsin (16,17), respectively. Since the production of $O_2^-$ by activated neutrophils is inhibited to a greater extent by TPCK than by TLCK, it has been suggested that the protease involved in the production of $O_2^-$ by human neutrophils is a "chymotrypsin-like" serine esterase (9,10). However, chloromethyl ketones are potent sulfhydryl group inhibitors (14,18). Thus, inhibition of $O_2^-$ production could be due to their inhibition of sulfhydryl groups. This study was designed to explore this possibility.

## MATERIALS AND METHODS

Reduced glutathione (GSH), phorbol myristate acetate (PMA), ferricytochrome C (horse heart, Type VI), superoxide dismutase (SOD, bovine blood, E.C. 1.15.1.1.), 5,5'-dithiobis - (2-nitrobenzoic acid) (DTNB), diisopropylfluorophosphate (DFP), L-1-tosylamide-2 phenylethylchloromethylketone (TPCK) and n-α-p-tosyl-L-lysine-chloromethylketone (TLCK) were obtained from Sigma Chemical Co., St. Louis, MO. TPCK was dissolved in dimethylsulfoxide (DMSO) and then diluted to desired concentrations with buffer before use. The concentration of DMSO on the final incubation medium was always less than 1.6%. Human neutrophils were isolated from venous blood of normal volunteers as described previously (19). The final preparation contained more than 95% pure neutrophils and was more than 96% viable as assessed by trypan blue dye exclusion (20). Low molecular weight (non-protein)-SH groups were measured by the procedure of Jocelyn (21). Neutrophils ($1.5 \times 10^7$) were incubated with or without inhibitors for 20 min. at 37°. After centrifugation at 800xg for 5 min. at 4°C, the cells were washed once with 10 ml cold modified Hanks' solution (22). They were then resuspended in 2 ml of de-ionized distilled water for 20 min. at room temperature, followed by the addition of 1 ml of 0.2M phosphate buffer pH 6.8. The tubes were centrifuged at 800xg for 5 min. at 4°C to remove the cell debris. An aliquot (2.8 ml) of the supernate was pipetted into a 4 ml cuvette and 0.2 ml of 10mM DTNB (Ellman's reagent) was added. The absorbance at 412 nm was determined 2 min. after the introduction of DTNB. The concentration of -SH groups was calculated according to Ellman (23) using extinction coefficient of 13600 OD/mole/cm.

Glutathione (GSH) was determined using DTNB (24). Briefly, 0.5 ml of GSH (1mM) was incubated with 0.5 ml of buffer containing inhibitors for 10 min. at 37°C. An aliquot of the sample (0.4 ml) was then added to a 4 ml cuvette containing 2 ml of phosphate buffer (0.15 M, pH 7.4) and 0.25 ml of DTNB (10mM). After mixing, the absorbance at 412nm was determined within 30 seconds.

Superoxide production by neutrophils was measured by SOD-inhibitable reduction of ferricytochrome C according to Babior et al (25). Neutrophils ($1.5 \times 10^7$) were incubated in the presence or absence of inhibitors and/or GSH for 20 min. at 37°C. Ferricytochrome C (50 nM) and PMA (0.1 ug/ml) with or without SOD (200U/ml) were then added in a final volume of 1.5 ml in modified Hanks' solution. After incubation for 20 min at 37°C, the reaction mixtures were passed through a 0.22 um Millipore filter. Ferricytochrome C reduction was determined by measuring the absorbance of the filtrate at 550 nm. The difference of absorbance at 550 nm in the presence and absence of SOD was taken as a measurement of superoxide.

Statistical differences were determined using Student's t test for independent means (26).

## RESULTS

As shown in Table 1, TPCK and TLCK markedly inhibited the production of $O_2^-$ by PMA-stimulated neutrophils. However, TPCK was a much more potent inhibitor than TLCK, consistant with previous reports (9,10). DFP at similar concentrations had no effect. A previous study has reported that DFP inhibits neutrophil superoxide production only at a concentration much higher than those shown in Table 1 (10).

Since TPCK and TLCK are also potent inhibitors of -SH groups (14,18), and since other -SH group inhibitors can inhibit $O_2^-$ production by neutrophils (27), the effect of these inhibitors on non-protein -SH groups of neutrophils was studied. As shown in Figure 1, TPCK and TLCK inhibited -SH groups of human neutrophils. Again, TPCK was a more potent inhibitor than TLCK. In contrast, DFP had no effect, while n-ethylmaleimide (NEM), a general -SH group inhibitor, was more inhibitory than TPCK.

TABLE 1

EFFECT OF CHLOROMETHYL KETONES AND DFP ON $O_2^-$ PRODUCTION BY PMA STIMULATED HUMAN NEUTROPHILS*

| INHIBITORS | | $O_2^-$ PRODUCTION (% CONTROL) | P VALUE (VS. CONTROL) |
|---|---|---|---|
| TPCK** | 0.01mM | 49.0±14.0(7) | <0.01 |
| | 0.05mM | 18.1±7.5 (10) | <0.001 |
| | 0.1mM | 10.4±5.1(7) | <0.001 |
| TLCK | 0.01mM | 49.2±15.9 (4) | <0.05 |
| | 0.1mM | 47.7±5.8(10) | <0.001 |
| DFP | 0.01mM | 110.3±22.8(3) | >0.6 |
| | 0.1mM | 94.6±15.3(5) | >0.7 |

*Neutrophils ($1.5 \times 10^7$) were preincubated with or without inhibitors for 20 min at 37°C. PMA was then added at a final concentration of 0.1ug/ml. Superoxide production was determined by SOD-inhibitable cytochrome C reduction. The results were expressed as mean ± SEM (% control). The control value was 0.326±0.053 (14) (O.D. absorbance at 550 nm).

**TPCK was dissolved in DMSO. The highest concentration of DMSO in the final medium was 1.6%. At this concentration, DMSO had no effect on $O_2^-$ production by PMA - Stimulated neutrophils (2 experiments). At the concentration used, TPCK and TLCK had no effect on the viability of neutrophils.

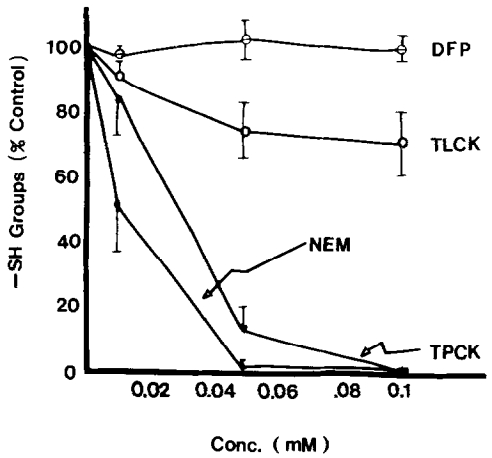


Figure 1. Effect of inhibitors on non-protein sulfhydryl groups of human neutrophils. Neutrophils ($1.5 \times 10^7$) were incubated with or without inhibitors for 20 min at 37°C. The cells were then washed, lysed and non-protein -SH groups determined using DTNB. The results were expressed as mean ± SEM (% control). The control value was 3.24 ± 0.16 (6) nmoles/$1x10^6$ cells.

Since most of the intracellular non-protein -SH groups are present as GSH, the effect of these inhibitors on GSH was then studied. As shown in Table II, TPCK, TLCK and NEM, but not DFP, inhibited GSH; and TPCK was a more potent inhibitor than TLCK.

The above results suggested that the inhibition of $O_2^-$ production by TPCK or TLCK could be in part due to their inhibition of neutrophil -SH groups. If this were the case, then one would expect that the inhibition

TABLE II

INHIBITION OF REDUCED GLUTATHIONE BY CHLOROMETHYLKETONES*

| | O.D. Absorbance (412 nm) | % Control |
|---|---|---|
| GSH Alone | 0.963±0.073 (3) | 100 |
| +TPCK | 0.246±0.018 (3) | 25.5 |
| +TLCK | 0.605±0.043 (3) | 62.8 |
| +DFP | 1.103±0.035 (3) | 114.5 |
| +NEM | 0.322±0.023 (3) | 33.4 |

*0.5 ml of GSH (1mM) was incubated with 0.5 ml of inhibitors (1mM) for 10 min at 37°. The residual content of GSH was then determined with DTNB. DMSO at concentrations up to 2.5% had no effect on GSH.

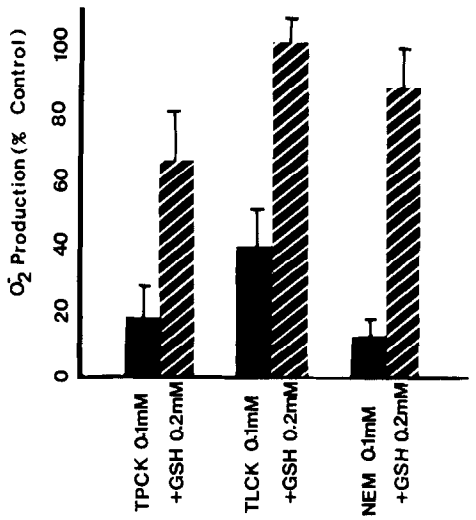


Figure 2. Effect of glutathione on the inhibition of $O_2^-$ production by chloromethyl ketones and NEM. Neutrophils ($1.5 \times 10^7$) were incubated with and without 0.1mM inhibitors and 0.2mM GSH for 20 min at 37°C. PMA (0.1 ug/ml) was then added and $O_2^-$ production determined. The results were expressed as mean ± SEM (% control).

of $O_2^-$ production could be prevented by GSH. As shown in Figure 2, GSH prevented the inhibition of $O_2^-$ production by PMA-stimulated neutrophils by TPCK, TLCK or NEM. GSH alone had no effect on the $O_2^-$ production by PMA-stimulated neutrophils (2 experiments, data not shown).

## DISCUSSION

Using potent inhibitors or synthetic substrates of serine esterases, several studies (1-11) have shown that protease activity may be essential for neutrophil chemotaxis, phagocytosis, degranulation and $O_2^-$ production. TPCK is an active-site histidine alkylating agent and is considered to be a specific inhibitor of chymotrypsin (15,16). In contrast, TLCK, also an active-site histidine alkylating agent, is considered to be a specific inhibitor of trypsin (16,17). Thus, they have been used to define the specificity of serine esterases. Using these two inhibitors, it has been suggested that the protease involved in the $O_2^-$ production by human neutrophils is a chymotrypsin-like protease (9,10). This conclusion is derived from the assumption that inhibition of $O_2^-$ production by TPCK and TLCK was due to their reactions with the putative protease. However, practically nothing is known about the

mechanism by which the protease activity is involved in these diverse neutrophil functions, nor do we know much about the nature and localization of the enzyme.

Considerable evidence in the literature suggests that TPCK and TLCK are potent inhibitors of -SH groups. Whitaker and Perez-Villasenor (18) have shown that inhibition of papain by TPCK and TLCK was due to their specific reaction with the sole -SH group (on cysteine residue No. 25) of papain. The rate of reaction of the chloromethyl ketones with papain is about 100 times faster than with trypsin or chymotrypsin, where they react with histidine (18). Rossman et al (14) showed that TLCK inhibited protein syntehsis by E. coli and that its inhibition could be prevented by reduced glutathione. Friedberg (28) also demonstrated that TLCK depleted the glutathione content of reticulocytes.

In this study, I demonstrated that the chloromethyl ketone derivatives, TPCK and TLCK, inhibited non-protein -SH groups and $O_2^-$ production by human neutrophils. TPCK was a better inhibitor of -SH groups as well as $O_2^-$ production. In addition, their inhibition of $O_2^-$ production by PMA-stimulated neutrophils could be prevented by GSH. These results strongly suggest that the inhibition of $O_2^-$ production by TPCK or TLCK is at least in part due to their inhibition of -SH groups. Because of the non-specific nature of many protease inhibitors, their use in a complex cellular system should be interpreted with caution.

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