Effect of maleimide derivatives, sulfhydryl reagents, on stimulation of neutrophil superoxide anion generation with concanavalin A

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Using maleimide derivatives with relatively rare side reactions, their effect on stimulation of O_2^- production by guinea-pig neutrophils with Con A was studied. Showdomycin, a very slowly penetrating agent, did not affect O_2^- production whereas NEM, a rapidly penetrating agent, markedly inhibited O_2^- production without interference with binding of Con A to cells. Particulate fractions from neutrophils stimulated with Con A showed markedly increased NADPH-dependent O_2^- production compared with fractions from unstimulated cells. Treatment of neutrophils with NEM before exposure to Con A inhibited the enhancement of NADPH-dependent O_2^- production of particulate fractions by Con A. However, particulate fractions from Con A-stimulated and unstimulated cells hardly exhibited reduced NADPH oxidase activity after direct exposure to NEM. Treatment of neutrophils with NEM after activation by Con A had no effect on NADPH-dependent O_2^- production of particulate fractions. These results indicate that NEM inhibits the activation process of the O_2^--generating enzyme, probably NADPH oxidase with Con A.

Chemical modification by maleimide derivative *Showdomycin* *N-Ethylmaleimide* *O_2^- generation* *O_2^--generating enzyme* *NADPH oxidase* *Lysozyme release* *Concanavalin A* *Guinea-pig neutrophil*

1. INTRODUCTION

Oxygen metabolites such as superoxide anions (O_2^-) and hydrogen peroxide are essential to the microbicidal function of neutrophils. Exposure to appropriate stimuli [1–9] is known to trigger the activation of a reduced pyridine nucleotide oxidase [10,11] in the plasma membrane [12,13] which catalyzes the one-electron reduction of molecular oxygen to O_2^-. The involvement of sulfhydryl groups in the O_2^--generating system of neutrophils is reported on the basis of the inhibition of O_2^- generation with sulfhydryl blocking reagents [9,14–16]. Under those modification conditions, various sulfhydryl groups could have been modified. However, functions other than O_2^- generation were not examined. Thus, the inhibitory mechanism of sulfhydryl reagents remains unelucidated.

In this communication, therefore, to elucidate which part of O_2^--generating system sulfhydryl groups are involved in, the effect of maleimide derivatives with relatively rare side reactions was studied on stimulation of guinea-pig neutrophil O_2^- generation with the lectin Con A and then on activation of the O_2^--generating enzyme, probably NADPH oxidase in a particulate fraction with Con A. The results suggest that sulfhydryl groups may be involved in the activation process of NADPH oxidase and that they may not be located on the cell surface membrane.

Abbreviations: Con A, concanavalin A; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline containing 1 mM $MgSO_4$ and 0.07 mM $CaCl_2$ (pH 7.4); SOD, superoxide dismutase; LDH, lactate dehydrogenase

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2. MATERIALS AND METHODS

Cytochrome *c* (type VI), superoxide dismutase (type I), Con A (type IV: from Jack beans), *Micrococcus lysodeikticus* (dried cells), catalase (C-40: from bovine liver), NADH (grade III) and FAD were purchased from Sigma, St Louis MO; β-NADPH, from Oriental Yeast, Tokyo; NEM from Wako, Osaka. Showdomycin was a gift from Shionogi Research Laboratory, Osaka. Con A (10 mg/ml) was dissolved in PBS and stored at −60°C. Acetylated cytochrome *c* was prepared essentially as in [17].

2.1. *Preparation of neutrophils and particulate fractions*

Guinea-pig neutrophils were isolated from the peritoneal cavity 13–15 h after injection of sterilized 0.12% glycogen in 0.9% saline as in [18]. The collected cells, which contained greater than 96% neutrophils, were resuspended at 2×10^7/ml in PBS.

Particulate fractions were isolated as in [19] with a slight modification. Briefly, neutrophil suspensions at 2×10^7/ml were incubated for 5 min at 37°C with 200 μg/ml Con A in PBS containing 1 mM $CaCl_2$. Treated cell suspensions were diluted with ~3 vols ice-cold PBS and centrifuged at 4°C for 8 min at 180 × *g*. Cells were resuspended in cold 0.34 M sucrose or PBS to 2.5×10^7/ml and sonicated immediately in an ice-water bath for 30 s at a setting of 74 W (Supersonic vibrator, model UR-150 P, Tominaga Works). Sonicates were centrifuged at 500 × *g* for 10 min at 4°C. Supernates were then centrifuged at 107000 × *g* for 30 min at 4°C. The pellets from the final centrifugation (particulate fractions) were resuspended in 0.34 M sucrose or PBS at a concentration equivalent to 5×10^7 cells/ml. Fractions from resting neutrophils were resuspended at a concentration equivalent to 1×10^8 cells/ml.

2.2. *Chemical modification of cells and particulate fractions*

Neutrophil suspensions at 2×10^7/ml were incubated with maleimide derivatives at 0°C for a defined time in PBS. The reaction was terminated by the addition of a 2-fold molar excess of cysteine followed by washing with a 10-fold excess of PBS. Modified neutrophils were resuspended at 2×10^7/ml in PBS. Control unmodified neutrophils were treated under the same conditions except that water was used instead of maleimide derivative solution.

Particulate fractions at a concentration equivalent to 2×10^7 cells/ml were treated with 100 μM NEM for 5 min at 0°C in PBS. After incubation, the reaction was terminated with a 2-fold molar excess of cysteine as above, and fractions were washed once with a ~8-fold excess of PBS by centrifugation at 107000 × *g* for 30 min at 4°C. Fractions were resuspended in PBS at a concentration equivalent to 5×10^7 cells/ml (1×10^8 cells/ml with fractions from resting cells). Control fractions were treated with H_2O in exactly the same manner as with NEM.

2.3. *Measurement of superoxide anion release*

The production of O_2^- by cells was measured by SOD-inhibitable cytochrome *c* reduction as in [20]. Neutrophils (5×10^6/ml) were incubated for 10 min at 37°C with 50 μg/ml Con A in the presence of 60 μM ferricytochrome *c* with or without 20 μg/ml SOD with constant shaking in a total volume of 3.0 ml PBS containing 1 mM $CaCl_2$. The reaction was started with the addition of stimulating agent (15 μl) and terminated by placing the reaction tubes on ice. The cells were pelleted by centrifugation at 1870 × *g* for 5 min at 4°C. The absorbance at 550 nm of supernatant was measured.

NADPH-dependent O_2^- production by isolated particulate fractions was measured as reduction of acetylated cytochrome *c* as in [17] with some modifications. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 200 μM NADPH, 80 μM acetylated cytochrome *c*, 5 μg/ml catalase, 20 μM FAD, 0.02% Triton X-100 and 0.3–1.0 mg/2.5 ml particulate fractions. Half of the mixture was placed in the sample cuvette and half in the reference cuvette, which also contained 30 μg/ml SOD. The assay was performed at 20°C and was followed continuously at 550 nm on a Hitachi 220A spectrophotometer. Initial slopes were used for calculations.

2.4. *Measurements of enzyme release*

In a final volume of 1.5 ml, the reaction mixture contained 1.5×10^7 neutrophils and 50 μg/ml Con A in PBS containing 1 mM $CaCl_2$.

Neutrophils were preincubated for 5 min at 37°C, followed by addition of stimulus or solvent. After 30-min incubation, samples were iced and centrifuged at 1870 × g for 10 min at 4°C. Supernates and total cell mixtures that were treated with Triton X-100 at a final concentration of 0.2%, followed by sonication and centrifugation at 1870 × g for 10 min at 4°C were assayed for lysozyme and LDH as in [21,22]. Supernate enzyme was expressed as a percentage of the total.

3. RESULTS AND DISCUSSION

The effect of maleimide derivative pretreatment on Con A-stimulated O_2^- production was examined. As shown in fig.1A, showdomycin, a very hydrophilic, i.e., very slowly penetrating SH reagent [23], did not affect O_2^- production even when cells were pretreated for 30 min at 1 mM, suggesting that sulfhydryl groups involved in O_2^- production are not exposed on the cell surface. Since NEM is considerably lipophilic, i.e., rapidly penetrating [23], modification of cells was performed for 1 min at 0°C. As can be seen in fig.1B, the inhibitory effect of NEM pretreatment was dependent on the reagent concentration and NEM at 40 μM inhibited markedly the ability of Con A to stimulate O_2^- production. The effects of treatment with NEM at 0 or 37°C were compared and no differences in NEM effects on O_2^- production were observed.

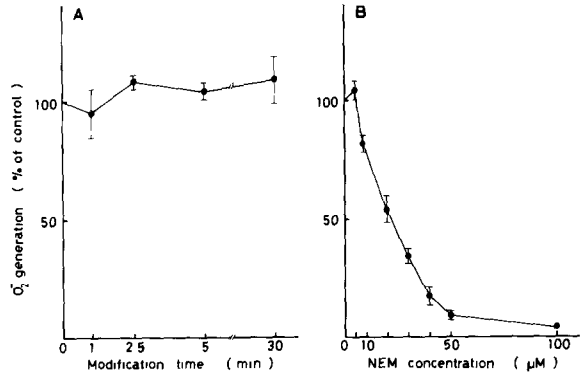

Fig.1. Effect of maleimide derivative pretreatment on O_2^- production by neutrophils exposed to Con A. Neutrophils were modified with 1 mM showdomycin at 0°C for indicated time periods (A) or with NEM at indicated concentrations for 1 min at 0°C (B). After maleimide derivative pretreatment, neutrophils were washed and then assayed for O_2^- production for 10 min in the presence of 50 μg/ml Con A. Values plotted are the means of 3 experiments with showdomycin and 5 experiments with NEM. The bars represent the SE. Each experiment was done in duplicate. Intact cells reduced 50.7 ± 14.0 nmol cytochrome *c*/10 min per 10^7 cells (mean ± SD, $n = 5$).

The inhibition by NEM of Con A-mediated O_2^- production could be caused by interference with binding of Con A to cells. If so, then another function of stimulus binding, the extracellular release of granule enzymes, should also be inhibited by NEM pretreatment. Therefore, the effect of NEM pretreatment on the release of granule lysozyme by neutrophils in response to Con A was studied. Expressed as mean ± SD ($n = 4$) of percent release (range given in parentheses), the data were as follows: untreated and NEM-treated cells in the absence of Con A, 12.4 ± 3.5 (7.9–16.2) and 10.9 ± 3.6 (6.6–15.4), respectively; untreated and NEM-treated cells in the presence of stimulus, 27.6 ± 35 (22.9–30.4) and 22.3 ± 2.9 (20–26.3). Thus, NEM pretreatment did not affect the exocytosis of lysozyme that was induced by Con A. The release of cytoplasmic LDH from cells in the absence and presence of stimulus was hardly affected. These results indicate that the inhibition of O_2^- production is not due to the NEM modification of the binding site for Con A.

To clarify the inhibitory mechanism of NEM, the effect of NEM pretreatment on activation of the O_2^--generating enzyme, probably NADPH oxidase, was studied by measuring its activity in a particulate fraction from NEM-pretreated neutrophils stimulated by Con A. Fig.2 shows that the stimulation with Con A increased the enzyme activity in the particulate fraction from intact cells whereas 40 μM NEM pretreatment markedly suppressed the increase in the activity by Con A stimulation. As seen in fig.3 however, the 5-min modification of particulate fraction with 100 μM NEM did not influence the O_2^--generating enzyme activity greatly, suggesting that the activated O_2^--generating enzyme is not susceptible to NEM modification at least under our conditions. Then, the effect of NEM treatment on neutrophils after activation by Con A was examined. As can be seen in fig.4, NEM treatment after activation of

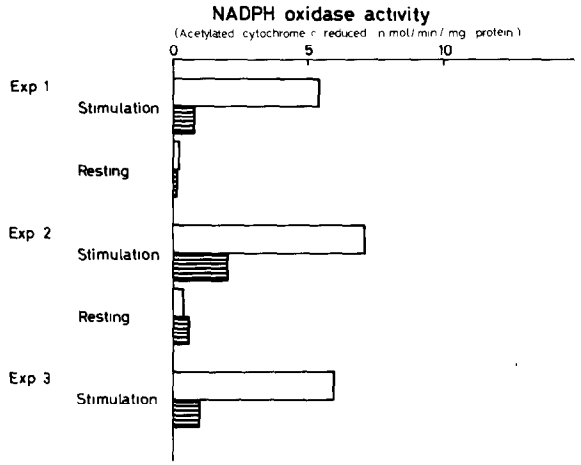

Fig.2. Effect of NEM pretreatment on NADPH-dependent O_2^- production of particulate fraction from Con A-stimulated neutrophils. Neutrophils (2×10^7/ml) were preincubated with 40 μM NEM (shaded bars) or H_2O (openbars) for 1 min at 0°C in PBS, then washed and resuspended in buffer. Resuspended cells were stimulated with Con A (stimulation) or with PBS (resting), then washed and resuspended in 0.34 M sucrose. Particulate fractions were made from sonicated cells by centrifugation at 107000 × g, suspended in 0.34 M sucrose and assayed for O_2^- release, as described in section 2.

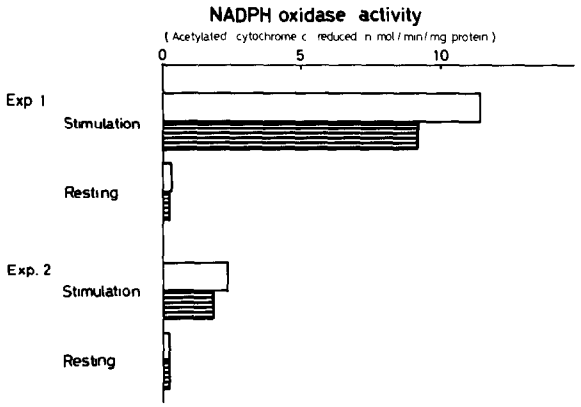

Fig.3. Direct effect of NEM on NADPH-dependent O_2^- production of particulate fraction from Con A-stimulated neutrophils. Neutrophils were stimulated with Con A (stimulation) or with PBS (resting), then washed and resuspended in PBS. After sonication and subsequent centrifugation at 107000 × g, particulate fractions were resuspended in PBS, modified with 100 μM NEM (shaded bars) or with H_2O (open bars) for 5 min at 0°C in PBS, washed and resuspended in PBS, as described in section 2. Resuspended particulate fractions were assayed for O_2^- release.

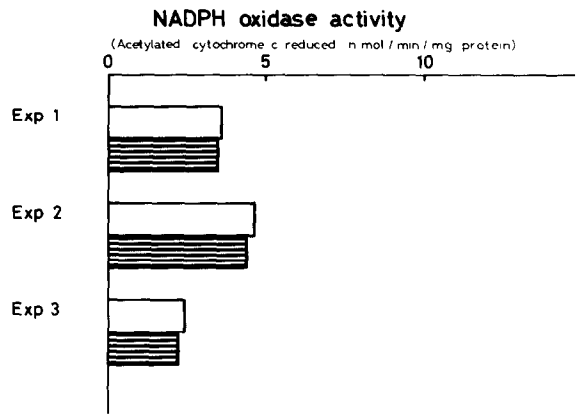

Fig.4. Effect of NEM treatment after activation by Con A on NADPH-dependent O_2^- production of particulate fractions. Neutrophils were stimulated with Con A for 5 min at 37°C, then washed and resuspended in PBS. Activated neutrophils (2×10^7/ml) were modified with 40 μM NEM (shaded bars) or H_2O (open bars) for 1 min at 0°C, then washed and resuspended in PBS. After removal of aliquots for O_2^- production by cells, cell suspensions were centrifuged, suspended in 0.34 M sucrose and sonicated. Sonicates were centrifuged at 107000 × g after removal of debris and nuclei and pellets were suspended in 0.34 M sucrose (particulate fractions) and assayed for O_2^- release. In exp.3, 100 μM NEM was used for a chemical modification of activated neutrophils.

neutrophils with Con A had little effect on increased NADPH-dependent O_2^- production of particulate fractions. The O_2^- production by cells was inhibited ~25% by NEM treatment after activation by Con A although about 85% inhibition was observed by NEM treatment before activation. Therefore, these above results seem to indicate that the inhibitory effect of NEM on the O_2^- production with Con A is due to the modification by NEM of sulfhydryl groups involved in the activation process of the O_2^--generating enzyme, probably NADPH oxidase, and that these sulfhydryl groups are not exposed on the cell surface membrane.

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