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A PROTEIN OF NEUTROPHIL GRANULES INTERFERES WITH ACTIVATION OF NADPH OXIDASE IN A CELL-FREE SYSTEM

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A soluble extract of neutrophil granules interfered with activation of the NADPH oxidase in a cell-free system . The extract had no effect on superoxide production by preactivated enzyme. The inhibitory activity was retained during dialysis and was lost upon exposure to proteinase K indicating that the active substance was a protein. The inhibitor exhibited a high stability at elevated temperatures . Chromatography of granules extract on ion exchangers implied that the inhibitor was a positively charged protein eluting from S Sepharose cation exchanger above 0.4M concentration of NaCl.

Superoxide production by stimulated neutrophils enables the cell to kill ingested microorganisms (1,2). The physiological significance of the superoxide generating NADPH oxidase is evidenced by susceptibility to infections of patients of chronic granulomatous disease (CGD) . This rare clinical syndrome is characterized by a defective oxygen radical production by patients phagocytes (3,4).

Since uncontrolled release of superoxide ions may damage body tissues, a careful regulation of the activity of NADPH oxidase is required. Latency of the enzyme in resting cells as well as pathways for deactivation of active NADPH oxidase in prestimulated PMN (5,6) represent different approaches for protection of body tissues against the effects of permanently active NADPH oxidase.

NADPH oxidase can be activated also in a cell-free system consisting of neutrophil or macrophage plasma membranes, cytosolic components and an unsaturated fatty acid (e.g.arachidonate) or SDS (7,8). In early reports on cell-free activation of NADPH oxidase in PMN , homogenates or unpurified cellular membranes were employed (7). Later, when only the light membrane fractions obtained by differential (9) or by density gradient centrifugation (10,11) were used for oxidase activation , specific activities improved . This observation suggested to us that in the granules fraction present in unpurified homogenates or membranes , an inhibitory substance might have been present. In the present report evidence for the existence of such an inhibitor of NADPH oxidase activation is presented.

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MATERIALS AND METHODS

Fractionation of neutrophils: Human neutrophils were isolated from fresh buffy coats by the standard procedure of dextran sedimentation and Ficoll density gradient centrifugation. Cells (10^8/ml) in 10mM potassium phosphate - (pH 7.0) buffered saline (PBS) containing 1mM EGTA , 7 mM phenylmethanesulfonylfluoride (PMSF) and 15 μg/ml leupeptin , were disrupted by sonication as described (11). Sonicates were centrifuged for 10 minutes at 800g and the pellets were discarded. Granules were sedimented 10 min at 10,000g and the supernatants were recentrifuged for 45 min at 204,000 g to give soluble cytosol and low density membranes . The membranes were resuspended in PBS / 0.34 M sucrose (sucrose-PBS).

NADPH oxidase activation and assay: Arachidonate-dependent activation was performed in two successive steps as described (11). Membranes and cytosol (1-2x10^6 and 2-4x10^6 cell equivalents respectively) in 0.1 ml sucrose-PBS containing 5 mM magnesium acetate and arachidonate (240-300 μM) were incubated 6 minutes at 30°C. After dilution with 0.7 ml sucrose-PBS / 0.2 mM NADPH / 80 μg cytochrome c , reduction rates of cytochrome c were measured at 550 nm before and after the addition of 30 μg of superoxide dismutase (SOD).

In most experiments oxidase was activated in a single step assay using SDS (50 μM) . The reaction buffer (0.8 ml) consisted of 10 mM pH 6.7 Hepes-buffered saline (HBS)/ 10 μM flavin adenine dinucleotide / 80 μM cytochrome c/ 1 mM EGTA . When column fractions eluted at high NaCl concentration were added to the activation mixture, controls of NADPH oxidase activity at identical salt concentration were run in parallel.

Extraction of granules: Granules pellet was resuspended in 20 mM Tris-HCl pH7.5 buffer/EGTA/leupeptin/PMSF at a density corresponding to 2X10^8 cell equivalents/ml and sonicated (3x20 sec). The sonicate was centrifuged 15 minutes at 27,000g and the supernatant representing the soluble granules extract containing 0.9-1.3 mg/ml protein was used as the source of the inhibitory material.

Ion exchange chromatography of the inhibitor : Granules extract (1-3ml) was applied to a 0.8x1.5 cm DE-52 cellulose (Whatman) column equilibrated with 20 mM Tris-HCl pH 7.5. The flow-through containing all the inhibitory activity was loaded on a 0.8x1.0cm S Sepharose (Sigma) column equilibrated with 20 mM Hepes pH 7.0 buffer. The column was washed with the equilibration buffer containing 0.15 M NaCl and eluted with a linear 0.15-0.8M NaCl gradient (20 ml total volume). Fractions of 1.0 ml were collected.

Protein concentration: was evaluated by the method of Lowry (12) . Columns fractions were monitored at 280nm .

RESULTS AND DISCUSSION

Inhibition of NADPH oxidase activation by granules extract. Arachidonate-promoted cell-free activation of the NADPH oxidase was carried out in two steps to permit separation of the activation and activity phases (11). When increasing amounts of the granules extract were present during the first (activation) phase , a dose dependent inhibition of enzymic activity was observed (Fig.1). Introduction of the inhibitory dose after completion of the activation (6 minutes) followed by an additional 6 minutes of incubation had no effect on superoxide production, indicating that the inhibitory substance interfered with activation step only and did not act as a superoxide scavenger. Since otherwise the experimental conditions at which the components of oxidase were exposed to the inhibitor were identical, this finding implied also

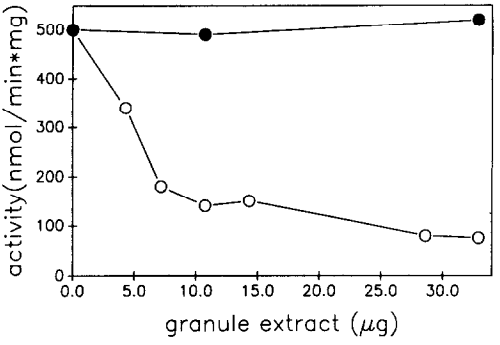

Fig.1. The effect of soluble granules extract on the activity of NADPH oxidase activated by arachidonate in a cell-free system.(o)-the extract was present during the initial step (6 minutes) of arachidonate-mediated activation ; (●)-extract was added after the initial 6 minutes preincubation of membranes , cytosol and arachidonate and incubated for an additional 6 minutes before final dilution.

that the latter did not act as a non specific protease . It follows that the inhibitory substance seems distinct from the NADPH oxidase-inactivating proteinase of azurophilic granules mentioned by Borregaard (13).

Properties of the inhibitory substance. Granules extract could be concentrated by ultrafiltration (Diaflo ultrafilter, Amicon, equipped with YM10 membrane) and when chromatographed on a Sephadex G-25 column, the active substance eluted in the void volume (results not shown); both findings indicated that the inhibitor was a macromolecule.

At pH 7.5 the inhibitor present in the extract of granules was not retained by a DE-52 cellulose anion exchange column . Flow-through of

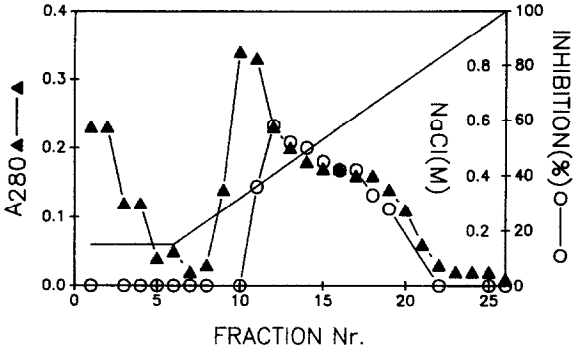

Fig.2. Chromatography of the NADPH oxidase inhibitory substance on a S Sepharose cation exchange column. Soluble extract of neutrophil granules (6.0 ml of 0.8 mg protein/ml) was loaded on a DE-52 cellulose column ; flow-through containing all the inhibitory activity (0.5mg/ml protein) was transferred to an S Sepharose column and eluted by salt gradient. 0.1 ml of each fraction was added to SDS-supported NADPH oxidase activation assay.

the DE-52 column containing all activity detected in the soluble extract , was loaded on a Sepharose S cation exchanger . The inhibitor was adsorbed by the resin and could be eluted as a broad peak between 0.4-0.7 M NaCl concentration (Fig.2).

The inhibitory substance (in the crude extract and in partially purified fractions from S Sepharose column) exhibited a high stability to heat: its activity was not reduced by 10 minutes incubation at 90°C (Table I). This property was taken advantage of in experiments devised to test whether it was susceptible to cleavage by proteinase K, namely, whether it was a protein . The granules extract was incubated 15 minutes with proteinase K (Merck). Before the resulting extract could be assayed for its effect on oxidase activation it was necessary to inactivate proteinase K, since the latter abolished activation of the oxidase (Table I). This was accomplished by exposure of the mixture of granule extract and proteinase K to 90° , a treatment which inactivates proteinase K (Table I). The extract treated with proteinase K lost its inhibitory activity, indicating that the active substance was a protein, cleaved by the protease.

In conclusion , we described a novel activity of neutrophil granules which interferes with activation of the NADPH oxidase in a cell-free system. The inhibitory substance is a positively charged , highly thermostable protein . It is conceivable that the inhibitory protein might have been defined previously but its regulatory function with respect to activation of the NADPH oxidase remained unrecognized .

The present study gives no indication regarding the mechanism of action of the inhibitor nor its exact location within the different subpopulations of granules (13,14) . It is noteworthy that Clark et al.,(15) and Borregaard (13) claimed that most of activatable, membrane-bound NADPH oxidase of resting , disrupted neutrophils is

Table I

Effects of heat and proteinase K on the capacity of the inhibitor to interfere with activation of NADPH oxidase

Additions	O_2^-(nmol/min*mg protein)
None	450
DE-52 flow-through (17.5 μg)	155
heated[a] DE-52 flow-through	160
proteinase K-treated[b], heated[a] DE-52 flow-through	400
proteinase K[c]	30
heated proteinase K[a]	400

[a]10 minutes at 90°C.
[b]70 μg protein in 0.1 ml 20 mM Tris-HCl pH 7.5 was incubated at room temperature with 40 μg proteinase K and heated for 10 minutes at 90 °C. 25μl of this mixture was added to the oxidase assay.
[c]10μg protein.

located in specific granules . This would suggest that the inhibitor described and partially charaterized in this study is confined to other compartments of the cell e.g. azurophilic granules.

The existence of a negative regulatory control of activation of the NADPH oxidase might have been anticipated. Most constituents of granules exhibit however antimicrobial activity (14) ; in this respect , a possible antiinflammatory activity of a protein that interferes with activation of the superoxide generating pathway is unusual. Further studies will be necessary to define the nature of the inhibitor and the mode of its activity within the cell.

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