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GTP-dependent and -independent activation of superoxide producing NADPH oxidase in a neutrophil cell-free system

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GTP and GTP-γ-S enhanced several-fold the NADPH-dependent superoxide production induced by sodium dodecyl sulfate in a cell-free system of pig neutrophils consisting of the mebrane fraction and two cytosolic fractions separated by gel filtration. The enhanced activity was decreased by the addition of GDP in a dose-dependent manner, but 70% of the activity in the absence of GTP remained even at 1 mM GDP. Only one cytosol fraction besides the membrane fraction was required for the activation in the presence of GTP. The cytosol fraction was analyzed by chromatography on 2',5'-ADP agarose and two components responsible for the GTP-dependent and independent activation were separated. These results suggest that at least two pathways are available for the activation of superoxide production in the cell-free system of pig neutrophils.

Superoxide; NADPH oxidase; SDS; Cell-free system; GTP; (Pig neutrophil)

1. INTRODUCTION

Neutrophils generate superoxide anion (O_2^-) during phagocytosis or when stimulated by a variety of stimuli [1,2]. The O_2^- production is considered to be catalyzed by the membrane-bound NADPH oxidase which is dormant in the resting state. Recently, the activating process has been studied in disrupted unstimulated phagocytes [3]. The oxidase in the homogenates of neutrophils can be activated by SDS [7–10] or fatty acids such as arachidonic acid and oleic acid [3–6,8–13]. Both cytosol and membrane fractions are necessary for the activation. Partial purification of the cytosol fraction has been performed [10–12,14,19], but the factors obtained are different from each other in number and molecular mass. The involvement of a guanine nucleotide-binding regulatory protein (G-protein) in the cell-free activating system has been suggested [8,11,15].

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Abbreviation: GTP-γ-S, guanosine 5'-*O*-(3-thiotriphosphate)

We have previously shown that the cytosol fraction of pig neutrophils is composed of two factors (C_1 and C_2) which are separated by gel filtration and exhibit synergy for the SDS-dependent activation of oxidase [14]. This report describes the further characterization of the cytosol factors and showed that there are at least two, the GTP-dependent and independent, pathways leading to the activation of the NADPH oxidase in the cell-free system of pig neutrophils.

2. MATERIALS AND METHODS

Sephadex G-200 and 2',5'-ADP Sepharose 4B were purchased from Pharmacia Fine Chemicals (Sweden). The following material was obtained from Sigma (USA): ferricytochrome *c*, superoxide dismutase (SOD), guanosine triphosphate (GTP), guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S). Other reagents were of analytical grade.

The membrane and cytosol fractions of pig neutrophils were prepared as previously described [14], except that a phosphate-buffered saline was replaced by a Hepes-buffered saline consisting of 131 mM NaCl and 10 mM Hepes, pH 7.0.

Superoxide production was measured by determining the rate of superoxide dismutase-inhibitable ferricytochrome *c* reduction at 550–540 nm using a dual-wavelength spectrophotometer (Hitachi 557) as described previously [14]. The protein concen-

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tration was measured by the method of Lowry et al. [16] or Bradford [17] with bovine serum albumin as the standard.

Chromatography of the cytosol fraction on Sephadex G-200 was carried out as previously described [14]. The cytosol fraction (15.8 mg in 10 ml) was applied to a Sephadex G-200 column (2.64 × 85 cm) equilibrated with Hepes-buffered saline, pH 7.0, at 4°C. Elution was carried out with the same buffer and fractions of 6 ml were collected. The O_2^- production was measured as described in the legend. Fractions between 36–40 and 52–59 are designated as C_1 and C_2 fractions, respectively.

The C_1 fraction was analyzed by chromatography on 2′,5′-ADP Sepharose 4B. The column of 2′,5′-ADP Sepharose 4B (7 ml) was equilibrated with a wash buffer consisting of 10 mM Hepes, pH 7.0, 0.34 M sucrose, 0.1 M NaCl and 2 mM NaN_3 at 4°C. The C_1 fraction (1.9 mg in 8 ml) was applied to the column and then the column was washed with the buffer at 6 ml/h for 6 h. Elution was carried out at a flow rate of 12 ml/h. The elution buffer consisted of 65 mM Hepes, pH 6.3, 0.34 M sucrose, 0.1 M NaCl and 2 mM NaN_3 with a linear gradient of increasing NADPH concentration.

3. RESULTS

The O_2^--producing activity was induced by SDS in a cell-free system consisting of the membrane fraction and two cytosolic factors (the C_1 and C_2 fractions) of pig neutrophils, as previously shown [14]. The SDS-induced activity was markedly enhanced by the addition of GTP or its nonhydrolyzable analogue GTP-γ-S, as shown in fig.1A. The concentrations required for half-maximal effects of GTP and GTP-γ-S were 2 μM and 500 nM, respectively, and the maximal effects were observed at 10 μM of both nucleotides. The membrane and cytosol fractions prepared from the cells treated with pertussis and cholera toxins both at 1 μg/ml showed no change in the potency to produce O_2^- in the presence and absence of GTP-γ-S. The activity increased by 10 μM GTP or GTP-γ-S was decreased by the addition of GDP in a dose-dependent manner with the half-inhibition concentration of 40 μM (fig.1B). The activity, however, was not completely abolished even at 1 mM GDP and about 70% of the activity without GTP-γ-S remained. The observations suggest that there may be two different, GTP-dependent and -independent, pathways to activate the NADPH oxidase in the cell-free system.

Fractionation of the cytosol by gel filtration on a Sephadex G-200 column gave peaks of the factors at about 300 kDa (C_1) and about 50 kDa (C_2) which exhibited synergy in the SDS-induced O_2^- production (fig.2A). The low C_2 activity was

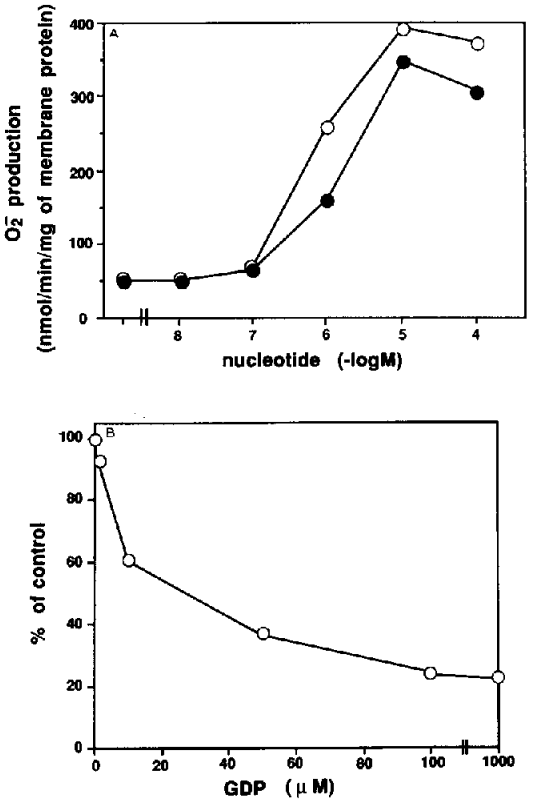

Fig.1. Influence of guanine nucleotides on SDS-induced O_2^- production in a cell-free system of pig neutrophils. (A) The O_2^- production was measured in the mixture of the membrane fraction (52 μg of protein), the C_1 fraction (48 μg of protein) and the C_2 fraction (92 μg of protein) in the presence of various concentrations of GTP (●) or GTP-γ-S (○) as described in section 2. (B) Effects of various concentrations of GDP on the O_2^- production in the presence of 10 μM GTP-γ-S. Assay conditions were the same as in A.

observed at a peak of about 1.3 kDa (C_3). When the SDS-induced O_2^- production was assayed by mixing single fraction of the eluates and the membrane fraction in the presence of 10 μM GTP-γ-S, only one peak which coincided with that of C_1 was observed (fig.2B). This indicates that C_1 contains at least two components, one needs C_2 and another needs GTP or GTP-γ-S besides the membrane fraction for the activation.

Table 1 shows the SDS-induced O_2^- production under various conditions. The membrane, C_1 or C_2 alone, or the mixture of the membrane and C_2 showed no detectable O_2^- production. The activity by the membrane plus C_1 was low and increased by

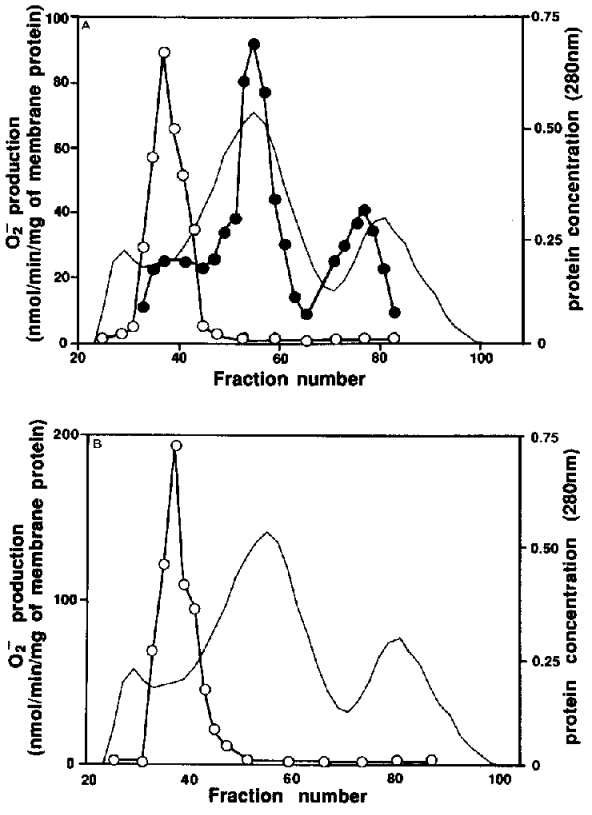

Fig.2. Gel filtration of the cytosol fraction on Sephadex G-200. Chromatography of the cytosol fraction on a Sephadex G-200 column was performed as described in section 2. The protein concentration of the eluate was monitored at 280 nm (——). (A) The activity of C_1 factor (○) was assayed with 0.2 ml of each fraction in the presence of C_2 fraction (0.2 ml of fraction 55) and the activity of the $C_2(C_3)$ factor (●) was assayed in the presence of C_1 fraction (0.2 ml of fraction 37). The SDS-induced O_2^- production by the mixture of factors C_1, $C_2(C_3)$ and the membrane fraction (55 μg of protein) is shown. (B) The SDS-induced O_2^- production was measured in the mixture of the membrane fraction (55 μg of protein) and single fraction of the eluates (0.2 ml) in the presence of 10 μM GTP-γ-S.

the addition of either C_2 or GTP-γ-S. Sum of the increased activities was essentially the same as the activity by the membrane plus C_1 in the presence of both C_2 and GTP-γ-S. This result supports the supposition that the C_1 fraction contains two components.

When C_1 was applied to a 2',5'-ADP Sepharose 4B column, the components responsible for the activation were bound to the gel. The components could be eluted by a linear gradient of NADPH as shown in fig.3. The O_2^--producing activity was

Table 1

SDS-induced O_2^- production in various conditions

Conditions	O_2^- production (nmol/min per mg of membrane protein)
Membrane	0
Membrane + C_1	17.9 ± 5.5
Membrane + C_2	0
Membrane + C_1 + GTP-γ-S	187.0 ± 41.7
Membrane + C_1 + C_2	70.9 ± 11.5
Membrane + C_1 + C_2 + GTP-γ-S	275.4 ± 31.7

The O_2^- production was measured in the presence and absence of 10 μM GTP-γ-S as described in section 2. The protein contents of the membrane fraction, the C_1 fraction (C_1) and the C_2 fraction (C_2) in the reaction mixture were 50 μg, 45 μg and 88 μg, respectively. The results are expressed as the means ± SD of three independent experiments

measured in the mixture of the membrane and the eluates in the presence of 10 μM GTP-γ-S (activity I) or the C_2 fraction (activity II), or both (activity III). The peaks of the activities I and II coincided with two peaks of the activity III. No activity was observed by incubation of the eluates and the membrane fraction without GTP-γ-S. The C_2 fraction did not bind to the gel. The observations show that the C_1 fraction contains two components in-

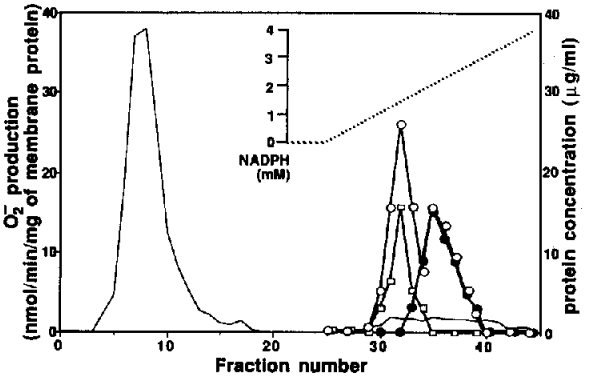

Fig.3. Demonstration of two components in the C_1 fraction by chromatography on 2',5'-ADP Sepharose 4B. Conditions of the chromatography are described in section 2. The O_2^- production was measured in the mixture of 0.2 ml of each eluate and the membrane fraction (55 μg of protein) in the presence of either the C_2 fraction (95 μg of protein) (activity I; ●——●) or 10 μM GTP-γ-S (activity II; □——□) or both (activity III; ○——○). The protein concentration of the eluate (——) was measured by the method of Bradford [17].

volved in the GTP-dependent and -independent activation of the O_2^- production in the cell-free system of pig neutrophils.

4. DISCUSSION

We show in this report that there are at least two pathways which lead to the activation of the NADPH-dependent O_2^- production in a cell-free system of pig neutrophils. The result might be physiologically significant, because distinct multiform pathways in intact cells have been reported to be involved in the activation mechanisms [2].

The observation that the two components in the C_1 fraction bound to a 2′,5′-ADP agarose could be eluted by NADPH, suggests a possibility that the components contain the NADPH-binding sites of the O_2^--producing oxidase and that the components translocate to the membrane during the activation process, because the oxidase activated in the cell-free system is restored in the membrane fraction [11,14,18]. Another possibility, however, should be considered. The membrane fraction from bovine neutrophils and the detergent extracts from the membranes of pig neutrophils are activated without the cytosol fraction by arachidonic acid and phosphatidic acid, respectively [18,20], indicating the presence of the NADPH-binding site in the membrane. The NADPH-binding component may localize both in the membrane fraction and in the cytosol fraction.

According to previous work [8,11] and our reports, a G-protein insensitive to pertussis and cholera toxin seems to be involved in the activation of O_2^- production in the cell-free system of neutrophils. Recently, the existence of G-proteins with lower molecular masses (22–26 kDa) in human neutrophils which are insensitive to the pertussis toxin has been demonstrated [21]. There remains a possibility, however, that the observed effects of guanine nucleotide are due to a Mg^{2+} GTP-dependent system other than a G-protein, such as tubulin or one of the microtubule associated proteins [22]. GTP and GTP-γ-S possibly bind to the component in the membrane fraction or to one of the components in the C_1 fraction responsible for the GTP-dependent activation. Doussiere et al. [18] have reported that GTP-γ-S interacts primarily with the cytosol factor of bovine neutrophils.

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REFERENCES

[1] Babior, B.M. (1984) J. Clin. Invest. 73, 599–601.
[2] Rossi, F. (1986) Biochim. Biophys. Acta 853, 65–89.
[3] Bromberg, Y. and Pick, E. (1984) Cell. Immunol. 88, 213–221.
[4] Heyneman, R.A. and Vercauteren, R.E. (1984) J. Leukocyte Biol. 36, 751–759.
[5] McPhail, L.C., Shirley, P.S., Clayton, C.C. and Snydermann, R. (1985) J. Clin. Invest. 75, 1935–1939.
[6] Curnutte, J.T. (1985) J. Clin. Invest. 75, 1740–1743.
[7] Bromberg, Y. and Pick, E. (1985) J. Biol. Chem. 260, 13539–13545.
[8] Shifert, R. and Schultz, G. (1987) Eur. J. Biochem. 162, 563–569.
[9] Cox, J.A., Yeng, A.Y., Bromberg, P.M. and Tauber, A.I. (1987) J. Immunol. 138, 1884–1888.
[10] Clark, R.A., Leidal, K.G., Pearson, D.W. and Nauseef, W.M. (1987) J. Biol. Chem. 262, 4065–4074.
[11] Gabig, T.G., English, D., Akard, L.P. and Schell, M.J. (1987) J. Biol. Chem. 262, 1685–1690.
[12] Curnutte, J.T., Kuver, P. and Scott, P.J. (1987) J. Biol. Chem. 262, 5563–5569.
[13] Tanaka, T., Kanegasaki, S., Makino, R., Iizuka, T. and Ishimura, Y. (1987) Biochem. Biophys. Res. Commun. 144, 606–612.
[14] Fujita, I., Takeshige, K. and Minakami, S. (1987) Biochim. Biophys. Acta 931, 41–48.
[15] Ligeti, E., Doussiere, J. and Vignais, P.V. (1988) Biochemistry 27, 193–200.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[18] Doussiere, J., Pilloud, M.C. and Vignais, P.V. (1988) Biochem. Biophys. Res. Commun. 152, 993–1001.
[19] Sha'ag, D. and Pick, E. (1988) Biochim. Biophys. Acta 952, 213–219.
[20] Bellavite, P., Corso, F., Dusi, S., Grzeskowiak, M., Bianca, V.D. and Rossi, F. (1988) J. Biol. Chem. 263, 8210–8214.
[21] Bokoch, G.M. and Parkos, C.A. (1988) FEBS Lett. 227, 66–70.
[22] Purich, D.L., Terry, B.J., MacNeal, R.K. and Karr, T.L. (1982) Methods Enzymol. 85, 416–433.