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TWO CYTOSOLIC COMPONENTS OF THE NEUTROPHIL NADPH OXIDASE, P47-*PHOX* AND P67-*PHOX*, ARE NOT FLAVOPROTEINS

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SUMMARY: Two cytosolic proteins, p47-*phox* and p67-*phox*, have been shown to be essential components of the NADPH-dependent oxidase of human neutrophils, although the specific role of each of these proteins in the multicomponent electron transport complex is undetermined. The superoxide-generating activity of this oxidase can be reproduced in a cell-free system, combining cytosol and membranes from unstimulated neutrophils in the presence of fatty acid and NADPH. In the present studies, cytosol was treated with myristic acid, arachidonic acid, or sodium dodecyl sulfate in the absence of membranes and the resultant precipitate collected by centrifugation and analyzed. Both p47-*phox* and p67-*phox* precipitated in the presence of fatty acid. However, neither FAD nor FMN was localized in the precipitates, even though substantial amounts of p47-*phox* and p67-*phox* precipitated. These results suggest that neither p47-*phox* nor p67-*phox* is a flavoprotein and that neither, therefore, is the oxidase component which accepts electrons from NADPH.

The activity of superoxide (O_2^-)-forming NADPH-dependent oxidase of human neutrophils can be produced *in vitro* in a cell-free assay which combines membrane and cytosol from resting neutrophils in the presence of a variety of fatty acids (1,2). Activity of the NADPH-dependent oxidase depends on the presence of membrane-associated b-type cytochrome and

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cytosolic factors, p47-*phox* and p67-*phox*. The central importance of p47-*phox* and p67-*phox* is emphasized by immunochemical studies of patients with autosomal chronic granulomatous disease (CGD) whose cytosol lacks activity in the cell-free assay. The cytosols of these patients have been characterized and shown to be deficient in one or the other of these two components as determined by immunoblots with a polyclonal antiserum (B-1) which recognizes p47-*phox* and p67-*phox* (3,4). The cDNA for each of these cytosolic factors has been cloned and sequenced (5-7) and recent studies have demonstrated the agonist-related translocation of these factors from the cytosol to the membrane during activation of neutrophils (8).

Nonetheless it is not clear whether these two proteins are activating factors or catalytic components of the NADPH-dependent oxidase complex. However, it has been proposed that a cytosolic factor is the NADPH-binding site of the oxidase system (9-15). If one of the cytosolic components is the NADPH-binding site of the electron transport chain, it could well be a flavin enzyme that accepts electrons from NADPH. Clark et al (8) have previously reported that p47-*phox* and p67-*phox* precipitate from cytosol in the presence of a variety of fatty acids and that this precipitation does not require addition of membranes. In the present experiments, the cytosol of human neutrophils was mixed with fatty acids in the absence of membranes, resulting in marked precipitation of p47-*phox* and p67-*phox*, and the distribution of flavin content measured without interference from a high background of membrane-bound flavins (16,17).

MATERIALS AND METHODS

Materials FAD, FMN, cytochrome c, superoxide dismutase (SOD), N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and arachidonic acid (AA) were purchased from Sigma (St. Louis, MO); myristic acid (MA) and diisopropyl fluorophosphate (DFP) were from Wako Pure Chem. (Tokyo); sodium dodecyl sulfate (SDS) and phenylmethylsulfonyl fluoride (PMSF) were from Nakarai (Kyoto). Other chemical reagents were of analytical grade.

Preparation of membrane and cytosol fractions Human blood neutrophils obtained by Dextran sedimentation were treated with DFP and disrupted in an ice-cold relaxation buffer minus ATP (pH 7.3) by sonication in the presence of PMSF, TLCK and 2 mM azide as reported previously (18). The post-

nuclear supernatant was centrifuged at 230,000 x g for 15 min at 2°C in a table-top ultracentrifuge (Beckman model TL-100). The membrane fraction (pellet) was rinsed once with the same buffer by centrifugation and suspended in an aliquot of ice-cold 0.34 M sucrose containing 1 mM EGTA and 10 mM PIPES (pH 7.3) at 5 x 10^8 cell equivalents (CE) per ml. The supernatant was centrifuged at 480,000 x g for 15 min at 2°C to eliminate any membrane contamination.

Assay of NADPH-dependent oxidase activity NADPH-dependent O_2^- forming activity was measured at 25°C by recording the reduction of cytochrome c at 550-540 nm in a Hitachi dual wavelength spectrophotometer model 556. The reaction mixture (0.1 ml) contained 65 mM K-Na-phosphate buffer (pH 7.0), 0.17 M sucrose, 1 mM $MgCl_2$, 1 x 10^7 CE of membranes and 2 x 10^7 CE of cytosol. The reaction contents were mixed with various concentrations of fatty acids or appropriate amounts of ethanol as solvent control, and preincubated for 4 min at 25°C. Then it was diluted with the same buffer and mixed with 50 μM cytochrome c (final volume; 0.8 ml). The reaction was started by addition of 0.1 mM NADPH.

Treatment of cytosol with fatty acids (8) Four samples of 1 ml of cytosol (2 x 10^8 CE) were incubated with 1.9 mM MA, 0.43 mM AA, and 0.95 mM SDS, and 1.25% ethanol, respectively, for 5 min at 25°C, and then centrifuged at 480,000 x g for 15 min. The precipitates were resuspended to the original volume in relaxation buffer. The supernatants were tested for residual activity in the cell-free oxidase system by their addition in place of untreated cytosol.

Immunoblots with B-1 antiserum Immunoblotting was carried out as reported previously (3, 8). Proteins in samples (1 x 10^5 CE) were separated by SDS-PAGE and then electroblotted onto nitrocellulose sheets which were exposed sequentially to blocking reagent and a 1:200 dilution of B-1 antiserum. The two proteins of 47 and 67 kDa were detected with horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin G, *o*-dianisidine and 4-chloro-1-naphthol as substrates.

Assays of FAD and FMN The samples were treated with 10 % trichloroacetic acid at 0°C for 30 min and then centrifuged at 10,000 x g for 4 min in an Eppendorf 5412 centrifuge. The resulting extracts were adjusted to pH 2.6 or 7.4 for assays of FAD and FMN as reported previously (16,17).

RESULTS AND DISCUSSION

In initial studies we determined the amount of each fatty acid which maximally stimulated the NADPH-dependent O_2^- forming activity of the cell-free system. These amounts of myristic acid (MA), arachidonic acid (AA), and sodium dodecyl sulfate (SDS) were 200, 46, and 100 nmol per 2 x 10^7 CE of cytosol, respectively. Cytosol fraction was mixed with

these amounts of fatty acids in the absence of membranes, resulting in the formation of precipitates. Following centrifugation the supernatants lost most of the ability to induce NADPH-dependent O_2^- production in the cell-free system; the residual activity in the supernatant of MA-treated cytosol is 1% of that of control and no remaining activity in the cytosol treated with AA or SDS. Immunoblots using B-1 antiserum, which recognizes p47-*phox* and p67-*phox*, demonstrated that both cytosolic components were markedly precipitated by these fatty acids (Fig. 1). p67-*phox* was found exclusively in the precipitates but negligible in the supernatants and p47-*phox* precipitated almost completely with SDS, about 80% with AA, and about 50 % with MA. Both components were present in the supernatant but not the precipitate after addition of ethanol as the solvent control.

The distribution of flavins between the supernatant and precipitate fractions was examined. The data in Table 1 show that nearly all the FAD and FMN (92-96%) remained in the supernatants after precipitating p47-*phox* and p67-*phox* with the three fatty acids. The addition of exogenous FAD (10-100 nM) to the cytosol prior to fatty acid-treatment failed to demonstrate specific localization of FAD in the precipitates and the total FAD was recovered in the supernatants after fatty acid treatment (data not shown).

These results suggest that neither p47-*phox* nor p67-*phox* is a flavoprotein; and that neither therefore contains

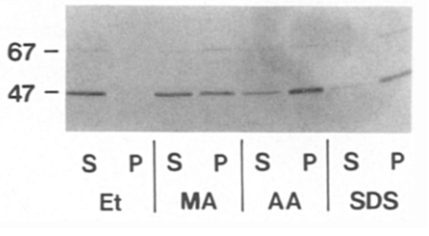

Fig. 1. Fatty acid-precipitation of p47-*phox* and p67-*phox* from cytosol.

Cytosol (2 x 10^7 CE) was treated with ethanol (Et) as a control, or 200 nmol of myristic acid (MA), 48 nmol of arachidonic acid (AA), or 100 nmol sodium dodecyl sulfate (SDS) for 5 min at 25°C, and then centrifuged to separate supernatant (S) and precipitate (P) fractions. Fractions (1 x 10^5 CE) were subjected to immunoblotting with B-1 antiserum.

Table 1. Flavin content of human cytosol treated with fatty acids

Treatment	Fraction	FAD (pmol)	FMN (pmol)	Total flavin (pmol)	% of total
Et	sup	233.0	39.0	272.0	92.4
	ppt	18.1	4.3	22.4	7.6
MA	sup	238.0	41.0	279.0	93.5
	ppt	14.0	5.3	19.3	6.5
AA	sup	227.0	46.5	273.5	95.1
	ppt	10.7	3.3	14.0	4.9
SDS	sup	235.0	48.6	283.6	95.9
	ppt	9.8	2.4	12.2	4.1

sup, supernatant; ppt, precipitate. Flavin content per 2 x 10^8 CE of cytosol. The supernatants and precipitates were the same as for Fig. 1.

the site that accepts electrons from NADPH. As reported previously, plasma membranes of neutrophils contain very similar amounts of flavins in resting and stimulated cells (16,17). Moreover, we recently found that a redox component, possibly a flavin enzyme, catalyzing the electron transfer from NADPH to cytochrome b-558 may be localyzed in the plasma membrane rather than the cytosol (H. Fujii and K. Kakinuma, in preparation). Since the discovery of the cytosolic components, there have been proposals that one of them may be the NADPH-binding component of the oxidase system (11-15). However, even if one of the cytosolic factors has a nucleotide-binding site, it appears to be an activation factor modifying the oxidase system rather than an electron transport component. Alternatively, cytosolic oxidase components could, after translocating to the membrane (8), serve a catalytic function distal to a flavoprotein.

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REFERENCES

1. Heyneman, R.A. and Vercauteren, R.E.(1984) J. Leuko. Biol. 36, 751-759.
2. Bromberg, Y. and Pick, E. (1984) Cell. Immunol. 88, 213-222.
3. Volpp, B.D., Nauseef, W.M., and Clark, R. A.(1988) Science 242, 1295-1297.
4. Clark, R.A., Malech, H.L., Gallin, J.I., Nunoi, H., Volpp, B.D., Pearson, D.W., Nauseef, W.M. and Curnutte, J.T. (1989) New Engl. J. Med. 321, 647-652.
5. Volpp, B.D., Nauseef, W.M., Donelson, J.E., Moser, D.R. and Clark, R.A. (1989) Proc. Natl. Acad. Sci. USA 86, 7195-7199, 9563 (Corr.).
6. Lomax, K.J., Leto, T.L., Nunoi, H., Gallin, J.I. and Malech, H.L. (1989) Science 245, 409-412; 246, 987 (Corr.).
7. Leto, T.L., Lomax, K.J., Volpp, B.D., Nunoi, H., Sechler, J.M.G., Nauseef, W.M., Clark, R.A., Gallin, J.I. and Malech, H.L. (1990) Science 248, 727-730.
8. Clark, R.A., Volpp, B.D., Leidal, K.G. and Nauseef, W.M. (1990) J. Clin. Invest. 85, 714-721.
9. Sha'ag, D. and Pick, E. (1988) Biochim. Biophys. Acta 952, 213-219.
10. Ishida, K., Takeshige, K., Takasugi, S. and Minakami, S. (1989) FEBS Lett. 243, 169-172.
11. Sha'ag, D. and Pick, E. (1990) Biochim. Biophys. Acta 1037, 405-412.
12. Takasugi, S., Ishida, K., Takeshige, K. and Minakami, S. (1989) J. Biochem. 105, 155-157.
13. Smith, R.M., Curnutte, J.T. and Babior, B.M. (1989) J. Biol. Chem. 264, 1958-1962.
14. Smith, R.M., Curnutte, J.T., Mayo, L.A. and Babior, B.M. (1989) J. Biol. Chem. 264, 12243-12248.
15. Okamura, N., Babior, B.M., Mayo, L.A., Peveri, P., Smith, R.M. and Curnutte, J.T. (1990) J. Clin. Invest. 85, 1583-1587.
16. Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986) J. Biol. Chem. 261, 9426-9432.
17. Kakinuma, K., Fukuhara, Y. and Kaneda, M. (1987) J. Biol. Chem. 262, 12316-12322.
18. Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. (1983) J. Cell Biol. 97, 52-61.