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PORCINE POLYMORPHONUCLEAR LEUKOCYTE NADPH-CYTOCHROME *c* REDUCTASE GENERATES SUPEROXIDE IN THE PRESENCE OF CYTOCHROME b_{559} AND PHOSPHOLIPID

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SUMMARY: NADPH-cytochrome *c* reductase and cytochrome b_{559} were purified from the membrane fraction of phorbol myristate acetate-stimulated porcine polymorphonuclear leukocytes. The highly purified reductase oxidized NADPH and generated superoxide when combined with partially purified cytochrome b_{559} in the presence of phosphatidylcholine. In the same system, but under the anaerobic condition, the reductase was found to reduce cytochrome b_{559}.

Our previous papers (1-5) demonstrated that an NADPH-cytochrome *c* reductase existed in the plasma membrane and phagosomes of both guinea pig and porcine polymorphonuclear leukocytes (PMN). The biochemical and immunological properties of the enzyme so far examined were found to be similar to those of liver microsomal NADPH-cytochrome P-450 reductase (4,5). As no detectable cytochrome P-450 was found in PMN, any physiological electron acceptor(s) of the reductase in the cells has not been determined yet. In this paper, we report that NADPH oxidation and superoxide (O_2^-) generation were observed when the reductase was combined with a *b*-type cytochrome of PMN, cytochrome b_{559}, in the presence of phosphatidylcholine. A possible role of the reductase in the respiratory burst is also discussed.

ABBREVIATIONS: PMN, polymorphonuclear leukocytes; O_2^-, superoxide; SOD, superoxide dismutase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DFP, diisopropyl fluorophosphate; PMA, phorbol myristate acetate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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MATERIALS AND METHODS

Materials: Ferricytochrome *c* (Type Ⅲ), superoxide dismutase (SOD), 2′,5′-ADP-agarose, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT) and dilauroyl phosphatidylethanolamine (synthetic) were obtained from Sigma Chemical Company, St. Louis, MO; Sephacryl S-300, *N*-amino-octyl-Sepharose and heparin-Sepharose from Pharmacia Fine Chemicals, Uppsala; diisopropyl fluorophosphate (DFP) from Kishida Chemical Co., Osaka; Triton X-100 and Triton X-114 from Nakarai Chemicals, Kyoto; NADPH and NADH from Oriental Yeast Co., Tokyo; DEAE-cellulose DE-52 from Whatman Ltd, Maidstone, Kent; Bio-Gel HTP from Nippon Bio-Rad Labs., Tokyo; phorbol myristate acetate (PMA) from LC Services Corp., New Boston, MA; dilauroyl phosphatidylcholine (synthetic) from Wako Pure Chemical Industries, Osaka.

Preparation of PMA-stimulated PMN: PMN were isolated from porcine blood as described previously (5). The cells ($2x10^8$ cells/ml) were stimulated with PMA as described in (3), and suspended in 50mM Tris-HCl, pH7.4, containing 0.34M sucrose, 1mM PMSF and 2mM DFP.

Purification of NADPH-cytochrome c reductase from PMN: The NADPH-cytochrome *c* reductase was highly purified from the membrane fraction of PMA-stimulated PMN as described in a previous paper (5). Analysis of the purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed substantially a single band (80kd). Its apparent turn over number of NADPH-dependent cytochrome *c* reduction on the basis of the FAD content was 2,000 min^{-1}.

Purification of cytochrome b_{559}: Cytochrome b_{559} was purified by combination of the methods described in (6-8) with some modifications. After solubilization of the NADPH-cytochrome *c* reductase in the presence of 0.2% Triton X-100 (5), residual pellet was suspended in 50mM Tris-HCl, pH7.4, containing 0.34M sucrose, 1% Triton X-114, 0.15M NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF and 2mM DFP, and kept at 4℃ for 12h for solubilization (7). Detergent phase separation of the extract was performed (7), and the partitioned cytochrome b_{559} was subsequently chromatographed on Bio-Gel HTP (7), Sephacryl S-300 (8), *N*-amino-octyl-Sepharose (6), 2′,5′-ADP-agarose (to remove a trace amount of the reductase) and heparin-Sepharose (6). All the resins were equilibrated with 50mM Tris-HCl, pH7.4, containing 20% glycerol, 0.2% Triton X-100, 0.15M NaCl, 0.1mM DTT, 1mM PMSF and 2mM DFP. The eluate of heparin-Sepharose was dialyzed against the equilibrated buffer, and used as partially purified cytochrome b_{559}. The purification of cytochrome b_{559} (5.0 nmol/mg of protein) was 88-fold with an overall yield of 5.0%, relative to the membrane fraction. The specific activity is comparable to those reported in (6-8). The cytochrome b_{559} thus purified showed a typical difference spectrum between the reduced and oxidized forms of the cytochrome (6-8) indicating the absence of any other type of cytochrome. An amount of cytochrome b_{559} was determined by measuring a difference spectrum between the reduced and oxidized forms, and calculated by employing a value of $21.6 mM^{-1} \cdot cm^{-1}$ for $\Delta \varepsilon_{559-540}$ (9).

Assays: NADPH-oxidizing activity was determined according to the method for assay of benzphetamine-dependent NADPH oxidation by liver microsomal NADPH-cytochrome P-450 reductase (5,10) except omission of benzphetamine and replacement of cytochrome P-450 by cytochrome b_{559}. Assay was carried out at 25℃ using a Hitachi Spectrophotometer 330. The complete reaction mixture contained 2.3pmol (based on FAD content) of the NADPH-cytochrome *c* reductase, 40pmol of cytochrome b_{559}, 30 μg of dilauroyl phosphatidylcholine, 50 μmol of HEPES buffer, pH7.4, 100 μg of deoxycholate, 15 μmol of $MgCl_2$, 0.1 μmol of EDTA and 0.1 μmol of

NADPH, in a final volume of 1.0ml. The reaction mixture was incubated at 25℃, and the reaction was initiated by addition of NADPH. The oxidation of NADPH was followed continuously at 340nm using an extinction coefficient of $6.11 mM^{-1} \cdot cm^{-1}$. O_2^--generating activity was determined in the same condition as that for assay of the NADPH-oxidizing activity except addition of 0.1 μmol of ferricytochrome *c*. An amount of O_2^- was determined by measuring SOD-inhibitable reduction of cytochrome *c* at A_{550} (11) using an extinction coefficient of $21.1 mM^{-1} \cdot cm^{-1}$ (12). Flavin content of the enzyme was determined by the method of Faeder and Siegel (13). Protein concentration was determined by using a protein assay reagent (Nippon Bio-Rad Labs., Tokyo) with bovine IgG as a standard.

Anaerobic condition: Anaerobic condition was achieved in a Thunberg-type cuvette. The sealed cuvette containing sample was cyclically evacuated and equilibrated with nitrogen gas which had been passed over a Deoxso purifier (Osaka Sanso Kogyo, Osaka) to remove a trace amount of contaminating oxygen via a closed three-way stopcock system. This cycle was repeated five times. After degassing on the final cycle, the reaction was initiated by addition of NADPH from a side arm of the cuvette.

RESULTS

Reconstitution of a O_2^--generating enzyme system: When highly purified NADPH-cytochrome *c* reductase was combined with partially purified cytochrome b_{559} in the presence of dilauroyl phosphatidylcholine, NADPH oxidation was found to proceed (Table I). When O_2 was omitted, no detectable NADPH oxidation occurred. Omission of the reductase, cytochrome b_{559} or phosphatidylcholine substantially resulted in no NADPH oxidation. To demonstrate whether O_2^- is generated or not, the reaction was performed in the presence of cytochrome *c*. The amount of O_2^- formed was 1.84 nmol/min, when estimated by measuring

Table I. **Requirements for NADPH oxidation**

Reaction mixture	NADPH oxidation (nmol/min)
Complete	1.05
- Cytochrome b_{559}	0.03
- NADPH-cytochrome *c* reductase	0.07
- Phosphatidylcholine	0.04
- NADPH	<0.01
- O_2 (anaerobic condition)	<0.01

NADPH oxidation was assayed as described in "Materials and Methods" except omission of indicated component from the reaction mixture.

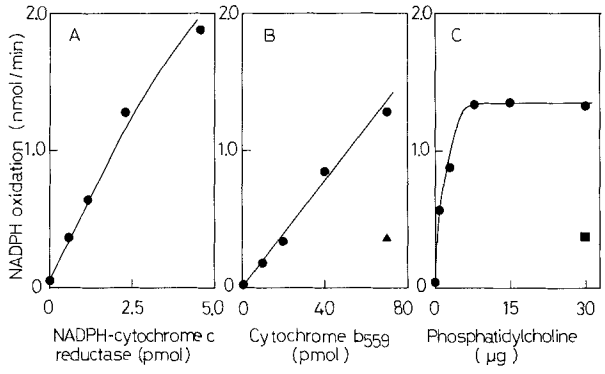

Figure 1. **Effects of concentration of NADPH-cytochrome *c* reductase, cytochrome b_{559} and phosphatidylcholine on NADPH oxidation.** NADPH oxidation was assayed as described in "Materials and Methods" except that the concentration of NADPH-cytochrome *c* reductase (A), cytochrome b_{559} (B) or phosphatidylcholine (C) was varied as shown. ▲, NADPH was replaced by the same concentration of NADH. ■, phosphatidylcholine was replaced by the same concentration of phosphatidylethanolamine.

SOD-inhibitable reduction of cytochrome *c*. Thus, the NADPH consumption may result in concomitant O_2^- generation in an approximate 1:2 molar ratio. Omission of cytochrome b_{559} or phosphatidylcholine resulted in direct reduction of cytochrome *c* by the reductase alone, indicating no O_2^- generation (data not shown). As both the NADPH-oxidizing and O_2^--generating activities were azide-insensitive (data not shown), they were not derived from any mitochondria enzyme system, if contaminating.

An apparent turn over number of the O_2^- generation on the basis of cytochrome b_{559} content in the enzyme system was 46 min^{-1}. This value is about one-fourth of that of the membrane fraction from PMA-stimulated PMN (200 min^{-1}). The specific activity of the enzyme system (230 nmol of O_2^- /min/mg of protein) was about ten times higher than that of the membrane fraction.

Figure 1 shows the effects of varying the concentration of each component on the NADPH oxidation. The rate of reaction was proportional to the concentration of the reductase, cytochrome b_{559} and phosphatidylcholine, respectively. The effect of phosphatidylcholine reached to a maximum at 15 μg (Fig. 1C). The replacement of dilauroyl phosphatidylcholine by dilauroyl phosphatidylethanolamine reduced the NADPH oxidation to 28%. The oxidation of NADH was only 28% of that of NADPH (Fig. 1B).

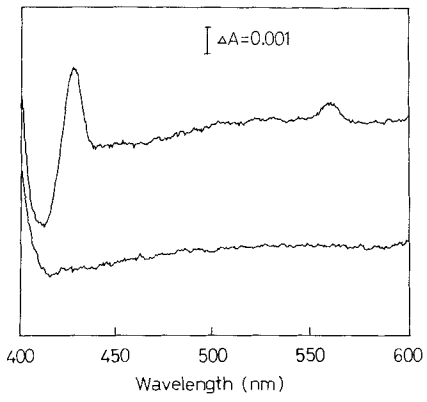

Figure 2. **Reduction of cytochrome b_{559} by NADPH-cytochrome *c* reductase.** Reaction was carried out anaerobically at 25℃ using Thumberg cuvettes as described in "Materials and Methods". Sample mixture (1.5ml) contained 11.4pmol of NADPH-cytochrome *c* reductase, 75pmol of cytochrome b_{559}, 45 μg of phosphatidylcholine, 75 μmol of HEPES buffer, pH7.4, 150 μg of deoxycholate, 22.5 μmol of $MgCl_2$, 0.15 μmol of EDTA and 0.15 μmol of NADPH. Reference mixture was identical with sample except omission of NADPH-cytochrome *c* reductase. After a base line was recorded, the reaction was initiated by addition of NADPH from a side arm. The upper line is a scan 8min after addition of NADPH. The lower line is a scan after reintroduction of air at 2h.

Reduction of cytochrome b_{559} by NADPH-cytochrme c reductase: We attempted to examine whether the reductase actually reduces cytochrome b_{559} or not. When the O_2^--generating enzyme system was incubated anaerobically, two peaks at 427 and 559 nm developed, which are characteristic to the reduced minus oxidized difference spectrum of cytochrome b_{559} (Fig. 2). The amount of cytochrome b_{559} reduced within 8min by the reductase was 51% of that fully reduced by dithionite, and scarcely increased even after prolonged incubation (2h). The spectrum thus developed disappeared immediately after reintroduction of air. When phosphatidylcholine was omitted from the enzyme system, the reduction of cytochrome b_{559} was not observed (data not shown). Thus, O_2^- may be generated by autooxidation of the cytochrome b_{559} reduced by the reductase.

DISCUSSION

PMN exhibit an enhanced production of O_2^- with a metabolic event known as the respiratory burst, which plays an important role in the

microbicidal activity (14). This O_2^- production is the result of activation of an NADPH oxidase embedded in the plasma membrane. The NADPH oxidase has been supposed to be a new electron transport chain composed of a flavoprotein (15,16) and cytochrome b_{559} (17,18). However, direct evidence for a biochemical entity of the NADPH oxidase, especially the flavoprotein involved, is still lacking due to its marked instability on various procedures for its purification. In the present study, we found first that PMN NADPH-cytochrome *c* reductase, which markedly resembles liver microsomal NADPH-cytochrome P-450 reductase (5), is able to reduce cytochrome b_{559}, resulting in O_2^- generation.

When the NADPH-cytochrome *c* reductase was combined with cytochrome b_{559} in the presence of phosphatidylcholine, NADPH oxidation as well as O_2^- generation were observed (Table I and Fig. 1). Moreover, the reductase was found to reduce anaerobically cytochrome b_{559} (Fig. 2). By analogy with cytochrome P-450 reduction by liver reductase (19), the enhancing effect of phosphatidylcholine on the O_2^--generating enzyme system reconstituted may be explainable by the following mechanism: Appropriate complex formation of the NADPH-cytochrome *c* reductase with cytochrome b_{559} through phosphatidylcholine is required for electron transfer from the reductase to the cytochrome. This possible mechanism may be supported by the finding that the NADPH-cytochrome P-450 reductase highly purified from liver microsomes exhibits a O_2^--generating activity when coupled with cytochrome b_{559} in the presence of phosphatidylcholine (data not shown).

As described in this paper, we succeeded first in reconstituting a O_2^--generating enzyme system by coupling the NADPH-cytochrome *c* reductase with cytochrome b_{559}. This result suggests strongly that cytochrome b_{559} may be a physiological electron acceptor of the reductase in PMN, and also that the reductase may be a putative flavoprotein of the respiratory burst NADPH oxidase.

On the other hand, the flavoprotein involved in the NADPH oxidase has been supposed to contain only FAD (14-16), differing from the reductase containing both FAD and FMN (5). In addition, several proteins differing from the reductase with regard to molecular weight have been proposed for candidates for the flavoprotein of the oxidase by others

:0-22). Therefore, irrefutable evidence for participation of the :ductase in the respiratory burst NADPH oxidase must await further ıvestigation.

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