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ESR signals from stimulated and resting porcine blood neutrophils

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The NADPH oxidase in neutrophils was specifically solubilized from membrane vesicles of porcine blood neutrophils and rapidly concentrated by immunoprecipitation with cross-reacting anti-P-450 reductase IgG. The precipitates from both myristic acid-stimulated and resting cells contained one third of the cytochrome *b*-558 and were slightly contaminated with myeloperoxidase. The immunoprecipitate from stimulated cells gave rhombic high-spin ESR signals of a heme at $g = 6.47$ and 5.49, which were insensitive to KCN, whereas the preparation from resting cells did not give these signals. The rhombic high-spin signals are discussed in view of the participation of cytochrome *b*-558 in the NADPH oxidase system.

Neutrophil; NADPH oxidase; Superoxide generating enzyme; Cytochrome *b*-558; ESR signal

1. INTRODUCTION

When exposed to bacteria and certain stimuli, neutrophils exhibit a KCN-insensitive respiratory burst that results in production of superoxide anion (O_2^- [1–4]. An NADPH oxidase found in neutrophil plasma membrane is the key enzyme in this respiratory burst, since stimulation of the cells induces rapid turnover of the oxidase [2,3]. This enzyme activity is not present in neutrophils of patients with chronic granulomatous disease (CGD) [5], indicating the importance of the oxidase system for bactericidal activity.

Several methods have been used in attempts to isolate the oxidase, but still little is known about its structure and reaction mechanism. Its active centers have been suggested to be flavin and *b*-type cytochrome (cytochrome *b*-558) from the following findings. (i) The enzymatic activity is inhibited by an FAD analogue, 5-carba-5-deaza-FAD [6]. (ii) A flavoprotein with an oxidation-reduction midpoint potential of −280 mV at pH 7.0 that probably reacts with NADPH in the stimulated state of the oxidase has been found in the neutrophil membrane fraction [7]. (iii) An O_2^--forming enzyme isolated by dye-affinity chromatography was shown to contain FAD [8]. (iv) An NADPH oxidoreductase containing an FAD protein, which was recently separated in our laboratory by isoelectric focussing on gel, seems to be responsible for the respiratory burst of the oxidase and has the ability to transfer electrons to a heme-protein (Kakinuma, K. et al., submitted). (v) There is no *b*-type cytochrome in neutrophils from the X-linked form of CGD [9,10]. (vi) Cytochrome *b*-558 was found to have a low midpoint potential of −245 mV [11]. (vii) Pyridine and imidazole, which bind to cytochrome *b*-558, inhibit the oxidase activity [12].

This paper reports a new method for the concentration of NADPH oxidase in an active form and studies on its ESR spectrum. The NADPH oxidase in neutrophil membranes was solubilized with

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detergents, and promptly concentrated by immunoprecipitation. Immunoprecipitation was achieved with anti-P-450 reductase IgG, which has been reported to cross-react weakly with the oxidase [13]. The precipitate obtained from stimulated neutrophils showed remarkable superoxide-generating activity, unlike that obtained from resting cells. The difference found in the ESR signals of the samples from stimulated and resting cells is discussed in relation to the NADPH oxidase system.

2. MATERIALS AND METHODS

2.1. *Purification of porcine P-450 reductase*

P-450 reductase activity was assayed by the method of Omura and Takesue [14]. A porcine liver microsome fraction was prepared and digested with trypsin by the methods of Iyanagi et al. [15,16]. The supernatant of the trypsin digest was applied to a DEAE-Sephacel column (Pharmacia) (3 × 10 cm) previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA, washed with the same buffer, and developed with a linear gradient (600 ml) of 0–0.5 M KCl in 1 mM EDTA/0.1 M potassium phosphate buffer (pH 7.7). Fractions of eluate with high reductase activity were combined and applied to a 2′,5′-ADP–Sepharose column (Pharmacia) (0.8 × 12 cm) equilibrated with 0.1 mM EDTA/0.1 M potassium phosphate buffer. The column was washed with 0.3 M potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, and then with the same buffer containing 0.2 mM $NADP^+$. Material was eluted with the buffer solution containing 10 mM $NADP^+$. The purified P-450 reductase obtained in this way appeared free from other proteins by analytical gel electrophoresis.

2.2. *Preparation of anti-porcine liver P-450 reductase IgG*

The purified P-450 reductase was dialyzed vs 0.9% NaCl in 10 mM potassium phosphate buffer (pH 7.5) for 4 h and concentrated by ultrafiltration (Amicon, PM-30 membrane) to a concentration of 2 mg protein/ml. Then 1 ml of the concentrated enzyme was emulsified with an equal volume of Freund's complete adjuvant (Iatron), and the emulsion (2.0 ml) was hypodermically injected into a male rabbit. Further injections of 1.4 ml of the same antigen fraction were given 5 times at 10-day intervals before preparation of immune serum. The IgG fractions from preimmune and immune sera were prepared by column chromatography on protein A-Sepharose CL-4B (Pharmacia). The antigen specificity of the IgG fractions was examined by the method of Ouchterlony and Nilsson [17]. The IgG fraction of immune serum formed a single precipitin line vs the purified P-450 reductase.

2.3. *Preparation of neutrophils and membrane vesicles*

Porcine blood neutrophils were separated by the method of Wakeyama et al. [18]. The cells were briefly incubated at 37°C with and without myristic acid [3] and centrifuged at 1000 × *g* for 5 min, the cell pellets being stored at −80°C for use in preparation of membrane vesicles [19]. The cells were thawed, disrupted by sonication, mixed with phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM, and centrifuged at 1000 × *g* for 20 min. The resulting supernatant was recentrifuged at 100000 × *g* for 60 min to obtain membrane vesicles.

2.4. *Solubilization of the NADPH oxidase*

NADPH oxidase was solubilized at 0–4°C. The membrane vesicles were suspended at a final concentration of 8 mg protein/ml in a solution of 0.2% (w/v) potassium deoxycholate, 0.25 M sucrose and 10 mM Hepes/NaOH buffer (pH 7.4). The mixture was stirred for 30 min, then centrifuged at 100000 × *g* for 45 min, and the pecipitate was suspended in a mixture of 37.5% (v/v) glycerol, 0.156 M sucrose and 6.25 mM Hepes/NaOH buffer (pH 7.4) with 80% by volume of the original deoxycholate mixture by homogenization in a glass homogenizer. Then 5% *n*-octyl-*β*-D-glucoside (Dotito) was added to the mixture to give a final concentration of 1% with stirring. The mixture was stirred for 30 min, centrifuged at 100000 × *g* for 60 min, and the supernatant fraction used for immunoprecipitation.

2.5. *Immunoprecipitation of the NADPH oxidase*

Immunoprecipitation was performed at 0–4°C. The solubilized fraction was diluted with an equal volume of a mixture of 0.1% octylglucoside, 30%

(v/v) glycerol and 10 mM Hepes/NaOH buffer (pH 7.4) (OGH buffer). After addition of the anti-P-450 reductase IgG fraction (final 0.1 mg protein/ml), the protease inhibitors 2 mM PMSF, 2 mM *N-p*-tosyl-L-lysine chloromethyl ketone, and 0.4–0.9 mM leupeptin were promptly added. The suspension was incubated at 2°C for 12 h, and then mixed with an anti-rabbit goat IgG fraction (Cappel; γ-chain-specific; final 0.2 mg protein/ml), and allowed to stand for 3 h at 2°C. The immunoprecipitate was collected by centrifugation at 50000 × *g* for 30 min, suspended in a small volume of OGH buffer and stored at −80°C.

2.6. *Determination of NADPH oxidase activity*

The O_2^--generating activity of the oxidase was assayed by measuring superoxide dismutase (37.5 μg protein/ml) inhibitable reduction of cytochrome *c* [20] in a Hitachi 556 dual-wavelength spectrophotometer. The assay mixture contained an aliquot of sample, 50 μM cytochrome *c*, catalase at 6 μg protein/ml, and 0.17 M sucrose in 65 mM sodium-potassium phosphate buffer (pH 7.0). The reaction was started by addition of 0.1 mM NADPH.

2.7. *Spectrophotometric determinations of cytochrome* b-*558 and myeloperoxidase*

Absorption spectra were recorded with a Hitachi 557 dual-wavelength spectrophotometer at 20°C. The contents of cytochrome *b*-558 and myeloperoxidase were determined from the difference spectra of sodium dithionite reduced minus oxidized samples using molar extinction coefficients of 21.6×10^3 (559–540 nm) [21] and 75.0×10^3 (472 nm) [22] $cm^{-1} \cdot M^{-1}$, respectively.

2.8. *ESR measurement*

ESR spectra were recorded in an X-band JES-FE 2XG spectrometer (JEOL), equipped with a sample dewar containing liquid helium. The conditions for measurements were as follows: microwave power, 5 mW; modulation amplitude, 20 G at 100 kHz; magnetic field, 1500 ± 1000 G; response, 1 s; sweep time, 4 min; amplitude, × 2500; temperature, 4.2 K.

2.9. *Other methods*

Myeloperoxidase of porcine neutrophils was purified by the method of Odajima [23]. The A_{430nm}/A_{280nm} ratio of the enzyme preparation used in this work was 0.5. Protein was determined by the method of Lowry et al. [24] with bovine serum albumin as a standard. Analytical polyacrylamide gel electrophoresis in the presence of SDS was carried out using the system reported by Laemmli [25].

3. RESULTS AND DISCUSSION

Results on solubilization and immunoprecipitation of NADPH oxidase of neutrophils are summarized in table 1. Membrane vesicles were pretreated with a low concentration of deoxy-

Table 1

Heme contents and NADPH oxidase activity

		Protein (mg)	Cyt.*b*-558 (nmol)	MPO (nmol)	NADPH oxidase activity[a]	Spec. act.[b]
Membrane vesicles	S	80.0	22.9	14.3	8650	378
	R	80.0	19.2	9.7	0	0
Solubilized supernatant	S	3.5	12.8	0.58	3270	255
	R	3.9	10.6	0.46	0	0
Immuno-precipitate	S	n.d.	6.9	0.36	1280	186
	R	n.d.	7.8	0.45	0	0

[a] nmol O_2^-/min

[b] nmol O_2^-/min per nmol cyt. *b*-558

Assays are described in section 2. Cyt., cytochrome; MPO, myeloperoxidase; S, preparation from stimulated neutrophils; R, preparation from resting neutrophils. n.d., not determined

cholate to remove peripheral membrane proteins, and then the membrane-bound NADPH oxidase was specifically solubilized with 1% octylglucoside. The solubilized supernatant contained 38% of the NADPH oxidase activity and 56% of the cytochrome *b*-558. Its contamination with myeloperoxidase, another heme-protein in neutrophils, was low (4%). The solubilized NADPH oxidase was precipitated by its cross-reaction with anti-P-450 reductase IgG. Anti-P-450 reductase IgG was used at a concentration of 0.1 mg protein/ml for immunoprecipitation, and had no inhibitory effect on the NADPH oxidase activity at up to 0.3 mg protein/ml. The coprecipitate contained 39% of the NADPH oxidase activity and 60% of the cytochrome *b*-558 of the solubilized supernatant fraction. The yields of cytochrome *b*-558 and myeloperoxidase from stimulated and resting cells were similar (table 1).

This immunoprecipitation is useful for obtaining concentrated NADPH oxidase in an active form. Furthermore, with membrane vesicles [7] and Percoll-isolated membrane vesicles [26], we have been unable to measure ESR spectra due to interference by contaminating materials (not shown), but these immunoprecipitation contained very small amounts of non-heme iron giving an ESR signal of about $g = 4.3$ [23] and of myeloperoxidase, and so weak ESR signals could be detected at low magnetic field without significant interference.

Fig.1a,b shows the ESR spectra of the immunoprecipitates prepared from stimulated and resting neutrophils, respectively. In both preparations, signals of hemes were detected only at low magnetic field. Fig.1c shows the ESR signals of myeloperoxidase purified from porcine neutrophils. The result shows that the signals of the immunoprecipitation at $g = 6.78$ and 5.12 (fig.1a,b) were due to the myeloperoxidase. The axial signal at $g = 6.00$ obtained with both preparations (fig.1a,b) might be attributable to cytochrome *b*-558 or contaminating hemoglobin derivatives. In contrast, only the preparation from stimulated cells gave signals at $g = 6.47$ and 5.49 (fig.1a). These signals seem to be attributable to the cytochrome *b*-558 associating with the NADPH oxidase in the stimulated state, since the electronic state of the other hemes contaminating the preparation would not be changed by generation of superoxide anion, except those of ESR-silent oxygen intermediates of myeloperoxidase.

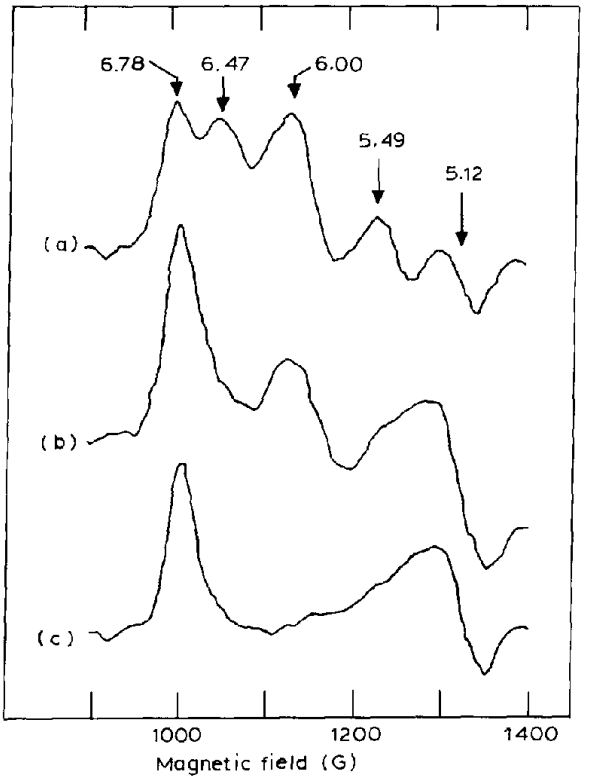

Fig.1. ESR signals from neutrophils. Conditions for ESR measurements were: microwave power, 5 mW; modulation amplitude, 20 G at 100 kHz; temperature, 4.2 K; magnetic field, 1500 ± 1000 G. (a) Immunoprecipitate from stimulated cells (2.0 μM cytochrome *b*-558); (b) immunoprecipitate from resting cells (2.7 μM cytochrome *b*-558); (c) purified myeloperoxidase (0.45 μM heme).

Next, we reduced both preparations with sodium dithionite and measured their ESR signals (not shown). No distinguishable signal was obtained, suggesting the absence of an iron-sulfur complex in the oxidase.

For further assignment, we examined the effect of 10 mM KCN on these high-spin signals (fig.2a,b). We found that KCN diminished the high-spin ESR signals at $g = 6.78$ and 5.12. This finding confirmed that these signals were due to myeloperoxidase, because the CN-ligated form of myeloperoxidase has been reported to lose its high-spin ESR signals [23]. The axial high-spin signal at

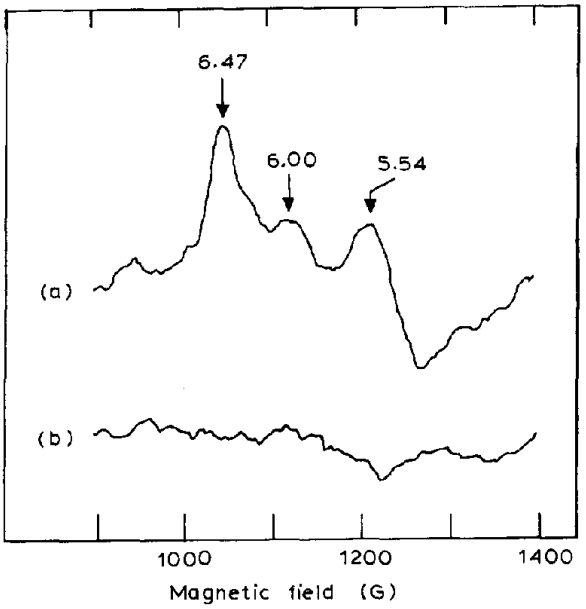

Fig.2. Effects of KCN on ESR signals from neutrophils. 10 mM KCN was added to each preparation and the mixtures incubated for 5 min at 20°C. Conditions for ESR measurements and cytochrome contents of each sample were as for fig.1. (a) Immunoprecipitate from stimulated cells; (b) immunoprecipitate from resting cells.

g = 6.00 was decreased by the addition of KCN, while the signals at g = 6.47 and 5.54 were KCN-insensitive. Although high-spin hemes are empirically thought to be KCN-sensitive, some membrane intrinsic heme-proteins are KCN-insensitive, such as the *b*-type cytochrome of the *bd*-type oxidase complex in *Escherichia coli* [27]. Therefore, the rhombic signals might be attributable to the cytochrome *b*-558 associated with KCN-insensitive NADPH oxidase in the neutrophil membrane fraction, and the oxidized cytochrome *b*-558 in the stimulated state is suggested to be in a high-spin state as for other terminal oxidases [28,29].

Recently, two groups have reported evidence for a molecular change in cytochrome *b*-558 following cell stimulation: Papini et al. [30] reported that a 31.5 kDa protein, probably cytchrome *b*-558, was phosphorylated after stimulation of the NADPH oxidase. Iizuka et al. [31] demonstrated using low-temperature absorption spectrophotometry that the main peak of the α-band of the cytochrome *b*-558 split into double peaks when the cells were stimulated anaerobically. In view of these findings, we propose that a change in the electronic state of cytochrome *b*-558 occurs following stimulation of the NADPH oxidase system.

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