Properties of NADPH Oxidase in Specific Granule-Rich Fraction
Prepared from Guinea Pig Neutrophils

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Both the plasma membrane-rich fraction and specific granule-rich fraction prepared from human neutrophil lysate by Percoll centrifugation have been reported to contain cytochrome b$_{558}$, a membrane activation factor for NADPH oxidase. In this study, the plasma membrane-rich fraction and specific granule-rich fraction of guinea pig neutrophils were prepared, and the abilities of both fractions to activate NADPH oxidase in a cell-free system consisting of either fraction, cytosol and arachidonate were compared. There was no difference in the $K_a$ value for NADPH between NADPH oxidase activated by specific granules or by plasma membranes. Such instability of specific granules was also observed on hypotonic- or deoxycholate-treatment. The inactivation by freeze-thawing was not suppressed by proteinase inhibitors, and gp91-phox, a large subunit of cytochrome b$_{558}$ was not degraded by freeze-thawing. Freeze-thawed specific granules did not affect the ability in plasma membranes, indicating the absence of an inactivating factor in specific granules. The increase in the amount of cytosol in the cell-free assay mixture did not compensate for the markedly decreased ability of freeze-thawed specific granules. Translocation of p47-phox, one of the cytosolic activation factors, to specific granules was not affected by freeze-thawing. We found that the ability of specific granules to activate NADPH oxidase was fragile, though it is unclear what is responsible for the instability, at present.

Key words NADPH oxidase; guinea pig; specific granule; freeze-thaw; cell-free activation

The most important function of neutrophils is to kill bacteria. The NADPH oxidase of neutrophils is activated by phagocytosis and produces superoxide anion (O$_2^-$), which then becomes microbialidal metabolites.[1] By phagocytosis and via various stimulators, cytosolic activation factors (p47-phox, p67-phox, p40-phox and p21-rac) associate with membrane activation factor (cytochrome b$_{558}$ composed of gp91-phox and p22-phox) to form an active NADPH oxidase complex in neutrophils.[2–4] NADPH oxidase is also activated by anionic amphiphiles, such as arachidonate and sodium dodecyl sulfate (SDS), in a cell-free system consisting of plasma membrane and cytosol.[5,6]

Cytochrome b$_{558}$ exists not only in plasma membranes but in specific granule membranes. Cytochrome b$_{558}$ of specific granules translocates to plasma membranes or phagocytic vesicles by phagocytosis or phorbol 12-myristate 13-acetate (PMA) stimulation.[7–9] Thus, it is proposed that specific granules are an intracellular pool of cytochrome b$_{558}$.[7,9] NADPH oxidase is also activated by arachidonate or by SDS in the cell-free system composed of cytosol and specific granules, instead of plasma membranes.[10,11] This indicates that cytochrome b$_{558}$ in specific granules can perform the functions of NADPH oxidase. Though there is more cytochrome b$_{558}$ in specific granules than in plasma membranes, little is known about how NADPH oxidase is activated in specific granules.[10,11] Thus, it is well worth studying the properties of NADPH oxidase activation in specific granules. In this study, we compared the ability to activate NADPH oxidase in specific granules with that in plasma membranes using the cell-free system.

MATERIALS AND METHODS

Materials Ferricytochrome c (horse heart type III), superoxide dismutase (SOD), phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin, and 1,4-piperazinediethanesulfonic acid (Pipes) were purchased from Sigma Chemical Co., PMA and arachidonate fromFunakoshi Pharmaceutical Co., (4-aminophenylene)methanesulfonyl fluoride (pA-PMSF) from Wako Pure Chemical Ind., Renaissance™ and Reflection™ from Du Pont, NADPH from Kohjin Co., anti-phosphotyrosine antibody PY20 from Transduction Laboratories, and molecular weight standards for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad Laboratories. Other chemicals were all of reagent grade from commercial sources. A rabbit polyclonal anti-p47-phox antibody was raised against the mouse p47-phox COOH-terminal peptide, PRPSSDLILHRCTESTKRLTSAV[12] and an antibody against human gp91-phox COOH-terminal peptide was a generous gift from Dr. J. D. Lambeth, Emory University Medical School, Atlanta, GA.

Guinea Pig Neutrophils Neutrophils were obtained from the peritoneal cavities of female guinea pigs of the Hartley strain as reported previously.[13] The content of neutrophils in the preparation was more than 90%.

Subcellular Fractionation of Neutrophils Resting neutrophils (7.5×10$^7$ cells/ml), suspended in a relaxation buffer (pH 7.3) containing 10 mM Pipes, 100 mM KCl, 3.0 mM NaCl, 3.5 mM MgSO$_4$, and 1.25 mM EGTA, were disrupted by sonication twice (2 A, 15 s) at 0 °C in the presence of protease inhibitors (200 μM PMSF, 20 μM PAA-PMSF, 2 μM Pepstatin, 2 μM Leupeptin). The lysate was centrifuged at 500×$g$, at 4 °C for 10 min to remove nuclei and unbroken cells, and the postnuclear supernatant (PNS) was fractionated by discontinuous Percoll gradient centrifugation according to the method of Borregaard et al.[7] The supernatant (cytosol fraction) and three visible bands named α, β, and γ in the gradient were collected, and ultracentrifuged at 100000×$g$ for 1 h to re-

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move Percoll, then the precipitated bands were resuspended in the relaxation buffer. Borregaard et al. described that the \( \alpha \), \( \beta \), and \( \gamma \) bands are azurophil granules, specific granules, and plasma membranes, respectively, in human neutrophils. Certainly, in our experiment, the \( \alpha \) band contained three-fourths of the total activity of myeloperoxidase, known as a marker enzyme of azurophil granules. Furthermore, the \( \beta \) and \( \gamma \) bands alone had no NADPH oxidase activity but showed abilities to activate the enzyme in the cell-free system when mixed with the cytosol fraction and arachidonate. On the other hand, the \( \gamma \) band, but not the \( \beta \) band, prepared from PMA-activated neutrophils, had NADPH oxidase activity. Thus, we used the \( \gamma \) band as the plasma membrane-rich fraction, and the \( \beta \) band as the specific granule-rich fraction in subsequent experiments.

**Freeze-Thawing of Plasma Membranes and Specific Granules** Half portions of the plasma membrane-rich fraction and specific granule-rich fraction prepared from neutrophils were frozen in a deep freezer at \(-80^\circ\text{C}\). Other portions were kept in an ice-cold water bath at 0 °C. After 16—24 h, frozen samples were gradually thawed on ice and used for cell-free NADPH oxidase activation.

**Treatment of Plasma Membranes and Specific Granules with Deoxycholate** Unfrozen plasma membrane-rich and specific granule-rich fractions were treated with given concentrations of deoxycholate for 30 min with gentle mixing at intervals of 10 min and 0 °C.

**Hypotonic Treatment of Plasma Membranes and Specific Granules** Unfrozen plasma membranes and specific granules were diluted seven-fold with distilled water. After 10 min at 0 °C, fractions were returned to the isotonic condition by the addition of concentrated relaxation buffer.

**Measurement of NADPH Oxidase Activity in a Cell-Free System** \( \text{O}_2^- \) produced by activated NADPH oxidase was continuously monitored at 25 °C on the basis of SOD-sensitive reduction of ferricytochrome \( c \).\(^{[14]} \) To activate NADPH oxidase, arachidonate was added to a reaction mixture consisting of 30 µg/ml plasma membrane protein or specific granule protein, 100 µg/ml cytosol protein, and 100 µM ferricytochrome \( c \) in the relaxation buffer (pH 7.3). To the reference cuvette, 100 units/ml of SOD was added. After 3 min at 25 °C, 200 µM NADPH was added and the increase in absorption at 550 nm corresponding to the reduction of ferricytochrome \( c \) was monitored. \( \text{O}_2^- \) production was calculated on the basis of an absorption coefficient of 21.0 M\(^{-1}\) cm\(^{-1}\). NADPH oxidase activity was expressed as \( \text{O}_2^- \) nmol/min/mg plasma membrane protein or specific granules protein.

**In Vitro Translocation Assay of p47-phox** A reaction mixture consisting of 100 or 400 µg/ml cytosol protein and 30 µg/ml plasma membrane protein in the relaxation buffer was incubated at 25 °C. Translocation was initiated by 40 µM arachidonate. After 3 min, the reaction mixture was carefully layered on the same volume of relaxation buffer containing 15% (w/v) sucrose to remove nonspecific precipitate of p47-phox caused by arachidonate, and was centrifuged at 10,000 x g and 4 °C for 2 h. The precipitated membrane fraction at the bottom of the centrifugation tube was subjected to immunoblotting.

**Western Blotting** The samples were mixed with concentrated SDS-PAGE sample buffer, then boiled and subjected to SDS-PAGE on 10% polyacrylamide gels using the Laemmli buffer system.\(^{[23]} \) The separated proteins were electrophoretically transferred onto a nitrocellulose sheet, probed with a 1:1000-fold dilution of antibodies against synthetic peptides from mouse p47-phox and human gp91-phox, and detected with a 1:3000-fold dilution of horseradish peroxidase-conjugated goat anti-rabbit Fc antibody using RenaissanceTM and ReflectionTM.

**RESULTS**

**Comparison of Kinetic Parameters of NADPH Oxidase Activated in the Cell-Free System in Plasma Membranes and Specific Granules** Ordinarily, plasma membrane and cytosol are used for a cell-free NADPH oxidase system. Moreover, it is reported that NADPH oxidase is activated even if specific granules are used instead of plasma membranes.\(^{[10]} \) But the activation in specific granules has been little investigated. Thus, we measured NADPH oxidase activity in a cell-free system consisting of arachidonate, cytosol and a plasma membrane-rich fraction or specific granule-rich fraction prepared with Percoll density gradient centrifugation\(^{[7]} \) from guinea pig neutrophils, and compared the kinetic parameters of the activated enzyme in both fractions (Table 1). The \( V_{\text{max}} \) of specific granules was about 2—3 times that of plasma membranes, but the \( K_m \) values for NADPH of both fractions were about the same. Figure 1 shows the optimum arachidonate concentrations for the activation of NADPH oxidase in specific granules and plasma membranes. Both opti-

<table>
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<tr>
<th>( V_{\text{max}} ) (O(_2) nmol/min/mg protein)</th>
<th>( K_m ) for NADPH (µM)</th>
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<tbody>
<tr>
<td>Plasma membranes</td>
<td>240±81</td>
</tr>
<tr>
<td>Specific granules</td>
<td>431±120</td>
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NADPH oxidase in a cell-free system consisting of 100 µg/ml cytosol protein and 30 µg/ml plasma membranes protein or 30 µg/ml specific granules protein was activated by the addition of 40 µM arachidonate. NADPH oxidase activity was measured as described in Materials and Methods. The values are the mean ± S.E. of three separate experiments.
Fig. 2. Effect of Freeze-Thawing on Cell-Free NADPH Oxidase Activation in Plasma Membranes and Specific Granules

The plasma membrane-rich fraction (PM) and specific granule-rich fraction (SG) were kept at 0°C or frozen at −80°C overnight, then the frozen samples were thawed. The samples (30 μg/ml protein) were used for NADPH oxidase activation in the cell-free system with 100 μg/ml cytosol protein and 40 μM arachidonic. NADPH oxidase activity was measured as described in Materials and Methods. The values are the mean±S.E. of 28–36 separate experiments.

Fig. 4. Effect of Deoxycholate on Cell-Free NADPH Oxidase Activation of Plasma Membranes and Specific Granules

Plasma membrane-rich (●) and specific granule-rich (●) fractions were treated with extraction buffer containing the indicated concentration of deoxycholate. The samples were kept at 0°C for 30 min with mild shaking at 10 min intervals. The samples (30 μg/ml protein) were used for NADPH oxidase activation in the cell-free system with 100 μg/ml cytosol protein and 40 μM arachidonate. NADPH oxidase activity was measured as described in Materials and Methods, and expressed as a percentage of 0% deoxycholate. The values are the mean±S.E. of three separate experiments.

Fig. 3. Effect of Hypotonic Treatment on Cell-Free NADPH Oxidase Activation of Plasma Membranes and Specific Granules

The plasma membrane-rich fraction (PM) and specific granule-rich fraction (SG) were diluted seven-fold with distilled water (hypotonic). After 10 min, the samples were returned to the isotonic condition by the addition of concentrated relaxation buffer. The samples (30 μg/ml protein) were used for NADPH oxidase activation in the cell-free system with 100 μg/ml cytosol protein and 40 μM arachidonate. NADPH oxidase activity was measured as described in Materials and Methods. The values are the mean±S.E. of three separate experiments.

Ultimate concentrations were 40 μM, but the activity in specific granules was markedly decreased at a slightly higher concentration, 50 μM. On the other hand, the activity in plasma membranes at 50 μM was the same as that at 40 μM. This implies a higher sensitivity of specific granules to the inhibitory effect of arachidonate.

Loss of Activation of NADPH Oxidase in the Cell-Free System by Freeze-Thawing In the cell-free system, it is possible to activate NADPH oxidase after the plasma membranes and cytosol have been frozen at −80°C. This is convenient when arranging a schedule of experiments. But, we found that freeze-thawed specific granules lost the ability to activate NADPH oxidase. Thus, we compared the abilities of the plasma membrane-rich fraction and specific granule-rich fraction, which were both frozen in a deep freezer at −80°C overnight and thawed just before use, or were kept in an ice-cold water bath at 0°C overnight (Fig. 2). The ability of plasma membranes was only slightly decreased by freeze-thawing, whereas that of specific granules was severely impaired.

The Susceptibility of NADPH Oxidase Activation Capability in Specific Granules to Various Membrane Perturbing Treatments Hypotonic treatment is known to slightly disrupt the membrane structure, so we maintained specific granule-rich and plasma membrane-rich fractions under hypotonic conditions for 10 min at 0°C. This treatment reduced the activation of NADPH oxidase of specific granules by about 25%, but did not affect plasma membranes (Fig. 3). A detergent, deoxycholate, influences membrane structure, and is used to solubilize NADPH oxidase activity from the plasma membrane of activated neutrophils. At 0.3%, deoxycholate impaired the abilities of both the specific granule fraction and the plasma membrane fraction. However, at 0.08%, the ability of plasma membranes actually exceeded that of the control, though the values varied between experiments (Fig. 4). It is known that, after subcellular fractionation, plasma membranes exist as vesicles, and binding sites for NADPH of the enzyme are directed inside the vesicles, where they are exposed by solubilization with deoxycholate, and thus NADPH oxidase activity is increased. On the other hand, the ability of specific granules was further reduced, depending on the deoxycholate concentration (Fig. 4).

Usually, a high concentration of KCl is used for the dissociation of proteins from membranes. The ability of specific granules was also more sensitive to the treatment with a high concentration of KCl (0.3—0.5 M) than that of plasma membranes (data not shown).

Effects of Proteinase on the Decrease in NADPH Oxidase Activation Though proteinase inhibitors were added to the relaxation buffer before subcellular fractionation, the inhibitors may be ineffective in plasma membrane-rich fraction and specific granule-rich fraction which were separated from cytosol by the fractionation and resuspended with the relaxation buffer without the inhibitors. To elucidate whether the proteinases affect the fragility of NADPH oxidase activation in the specific granule-rich fraction, we added fresh proteinase inhibitors to both the fractions after fractionation. The addition did not suppress the decrease in activation of

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Fig. 5. Effect of Freeze-Thawing on gp91-phox

gp91-phox, a large subunit of cytochrome b$_{558}$, was detected by Western blotting. Plasma membrane-rich fractions (PM; lanes 1, 2) and specific granule-rich fractions (SG; lanes 3, 4) were frozen at −80°C (lanes 2, 4) or kept at 0°C (lanes 1, 3) overnight, then the frozen samples (−80°C) were thawed. The samples had been centrifuged at 100,000×g and 4°C for 1 h, and the precipitates were boiled with SDS-PAGE sample buffer. The boiled samples were subjected to SDS-PAGE (10 μg protein/lane) and Western Blotting with anti gp91-phox antibody. A representative result of one out of three similar experiments is shown.

Fig. 6. Effect of Specific Granules on the Ability of Plasma Membrane to Activate NADPH Oxidase

The plasma membrane-rich fraction (PM), specific granule-rich fraction (SG), and a mixture of both fractions (PM + SG) were kept at 0°C or frozen at −80°C overnight, then the frozen samples were thawed. The samples (30 μg/ml protein) were used for NADPH oxidase activation in the cell-free system with 100 μg/ml cytosol protein and 40 μM arachidonate. NADPH oxidase activity was measured as described in Materials and Methods. The values are the mean ± S.E. of two separate experiments.

NADPH oxidase in specific granules caused by freeze thawing (data not shown).

Next, we analyzed quantitatively and qualitatively the alternation of gp91-phox, a large subunit of cytochrome b$_{558}$, by Western blotting. In both the plasma membranes and the specific granules, no quantitative alternation or decrease in the molecular weight of gp91-phox by degradation was observed (Fig. 5).

Involvement of Contents of Specific Granules in NADPH Oxidase Activation To investigate the possibility that some unknown inhibitory factor(s) in specific granules other than proteases is freed by membrane disruption, we mixed the specific granule-rich fraction and plasma membrane-rich fraction before freezing, and measured the ability of the freeze-thawed mixture (Fig. 6). The ability to activate the NADPH oxidase of the mixture was nearly equal to the sum of that of separately freeze-thawed fractions, which was markedly decreased in the specific granule-rich fraction and slightly decreased in the plasma membrane-rich fraction. This result indicates that the contents of specific granules did not suppress the ability of plasma membranes.

Association between Specific Granules and Cytosolic Activation Factors An association between cytosolic activation factors and cytochrome b$_{558}$ is necessary for activation of NADPH oxidase. It is possible that the membrane structure of specific granules was modified by freeze-thawing and as a result the affinity between cytosolic factors and cytochrome b$_{558}$ was decreased. Thus, we measured NADPH oxidase activation in the presence of various concentrations of cytosol (Fig. 7). The activity of the NADPH oxidase of the specific granule-rich fraction kept at 0°C was increased up to about 1200 nmol/min/mg protein, dependent on the increase in the cytosol protein concentration up to 400 μg/ml. In the case of specific granules kept at −80°C, the activation was also enhanced by the increase of cytosol concentration, but not to the extent of the non-frozen fraction. Because the loss caused by freeze-thawing in specific granules was not recovered by increasing the cytosolic protein concentration, the decline in activation is probably due to a decrease in the number of normally functioning cytochrome b$_{558}$ molecules, rather than a lowered affinity of the membrane factor for cytosol proteins.

Furthermore, we used Western blotting to investigate whether the translocation of p47-phox, one of the cytosolic activation factors, to the membrane was suppressed by...
freeze-thawing (Fig. 8). No notable effects of freeze-thawing on the translocation were observed in either plasma membranes or specific granules. These results suggest that freeze-thawing does not affect the association between specific granules and p47-phox.

DISCUSSION

Cytochrome b_{558}, a component of NADPH oxidase, is present in plasma membranes, though specific granules contain larger amounts of the cytochrome b. Recently, cytochrome b_{558} has been reported to be located on gelatinase-containing granules whose density is similar to that of specific granules. But there have also been reports that specific granules themselves contained gelatinase. It was also reported that cytochrome b_{558} resides on secretory vesicles, which were difficult to separate from plasma membranes by subcellular fractionation, and the cytochrome was easily translocated to plasma membranes during neutrophil purification. The location of cytochrome b_{558} on specific granules and plasma membranes is a subject of controversy. Cytochrome b_{558} content was decreased in specific granules and increased in plasma membranes by PMA and A23187 stimulation. Thus, it has been thought that cytochrome b_{558} in specific granule membranes exists as a pool and is supplied to plasma membranes when specific granules fuse with plasma membranes during phagocytosis.

Specific granules, as well as plasma membranes, express NADPH oxidase activity in cell-free systems on the addition of cytosol and arachidonate. But details as to the functions and characteristics of cytochrome b_{558} in specific granules are little known. Investigating the ability of cytochrome b_{558} to activate NADPH oxidase, we found the function of cytochrome b_{558} in specific granules to be much more fragile than that in plasma membranes. The ability of specific granules was easily lost by membrane perturbing treatments, such as freeze-thawing, hypotonic, deoxycholate-, and high concentration salt treatment. The ability was also reduced by 50μM arachidonate, which is slightly higher than the optimum concentration for cell-free activation. Loss of the ability was due to neither the degradation of gp91-phox, a large subunit of cytochrome b_{558}, nor to a reduction in the association between cytochrome b_{558} and p47-phox. At present, we cannot clarify how the ability of specific granules is impaired.

The discrepancy in the stability of cytochrome b_{558} function between specific granules and plasma membranes probably reflects conditions around cytochrome b_{558}. Though there are many possible explanations for the instability, the involvement of phospholipids is one of them. Phospholipids are known to play an important role with cytochrome b_{558} in NADPH oxidase activation. Functional membrane activation factor is reconstituted with purified cytochrome b_{558} and phospholipids, and the ability of the reconstituted factor depends on the type of phospholipid. Bjerrum et al. reported that, in human neutrophils, the phospholipid composition of specific granule membranes differs from that of plasma membranes, and the phosphatidylethanolamine (PC) content of plasma membranes is double the phosphatidylethanolamine (PE) content, but PE in specific granule membranes is three times more abundant than PC. In guinea pig neutrophils, we also preliminarily confirmed that PE content is greater than PC content in specific granules (data not shown). Thus, the difference in phospholipid composition between plasma membranes and specific granule membranes may explain the distinction in the stability to activate NADPH oxidase. Further studies, of course, are necessary to elucidate the exact mechanism of the fragility of the cytochrome b_{558} function in specific granule-rich fraction.

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