Respiratory Burst Oxidase Activation Can Be Dissociated From Phosphatidylinositol Bisphosphate Degradation in a Cell-Free System From Human Neutrophils

By Alexis E. Traynor, Patricia J. Scott, Anna L. Harris, John A. Badwey, Larry A. Sklar, Bernard M. Babior, and John T. Curnutte

Activation of the respiratory burst oxidase in cell-free preparations from 32P-labeled neutrophils was compared with changes in levels of radioactively labeled phosphoinositides in the same preparations. With membrane particles, treatment with sodium dodecyl sulfate (SDS) in the presence of cytochalasin led to activation of the oxidase without an alteration in levels of labeled phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylinositol 4-phosphate (PIP). Conversely, solubilization of the membrane particles with deoxycholate resulted in loss of nearly 98% of the radioactive PIP2 without activation of the oxidase. In this solubilized preparation, the oxidase could subsequently be fully activated by SDS in the presence of cytochalasin, even though the labeled PIP2 was almost totally depleted. Two PIP2-derived second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, as well as the protein kinase C activator phorbol myristate acetate (PMA), failed to activate the oxidase. These results suggest that in a cell-free preparation from human neutrophils, detergent-mediated activation of the respiratory burst oxidase is independent of changes in the levels of phosphoinositides or phosphoinositide-derived second messengers.

Several groups of investigators recently showed that the respiratory burst oxidase in resting neutrophil homogenates can be activated by addition of certain anionic detergents, including sodium dodecyl sulfate (SDS).1-10 To define the nature of that system better, we examined the fate of 32P-labeled phosphoinositides during the detergent-mediated activation of the oxidase. Our results strongly suggest that breakdown of the labeled pool of phosphoinositides is neither necessary nor sufficient for activation of the respiratory burst oxidase by anionic detergents.

MATERIALS AND METHODS

Materials. Cytochrome c (type VI), bovine erythrocyte superoxide dismutase, NADPH, phosphoinositides (bovine brain), phorbol myristate acetate (PMA), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 4-phosphate (PIP), inositol triphosphate, 1-isoproterenol, disisopropyl fluorophosphate (DFP), and potassium oxalate were obtained from Sigma, St Louis, SD. SDS was obtained from BioRad, Richmond, CA, and sn-1,2-dioctanoylglycerol was obtained from Calbiochem, La Jolla, CA. Stock solutions of 100 mmol/L dioctanoylglycerol and 3.24 mmol/L PMA were prepared in dimethylsulfoxide (DMSO), stored at -20°C, and then diluted with the same solvent the day of the experiment to working stock concentrations of 3.75 and 0.12 mmol/L, respectively. Percoll and Ficoll-Hypaque were purchased from Pharmacia, Piscataway, NJ. 32P-Labeled phosphoric acid (carrier-free, in water) was purchased from New England Nuclear, Boston, and Ecosint scintillation cocktail was purchased from National Diagnostics, New Brunswick, NJ. Chloroform, methanol, and acetone, all high-performance liquid chromatography (HPLC) grade, were obtained from American Scientific Products, Irvine, CA. Other reagents were the best quality commercially available and were used without further purification.

Labeling of neutrophils with 32P. Neutrophils were prepared by dextran sedimentation and purified over Ficoll-Hypaque as previously described.11 Cells (5 x 10^6 total) were washed once in buffer A (in mmol/L) NaCl 146, KCl 5, Na2HPO4 1.4, KH2PO4 0.22, MgCl2 1.03, MgSO4 0.28, CaCl2 1.16, and glucose 5.5, pH 7.4) at 4°C and resuspended at 5 x 10^6 cells/mL in buffer B with bovine serum albumin (BSA) [HEPES 30 mmol/L (pH 7.4) containing NaCl 110 mmol/L, KCl 10 mmol/L, MgCl2 1 mmol/L, glucose 10 mmol/L, and albumin 2 mg/mL]. To this suspension was added 32P-labeled

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phosphoric acid (HCl-free, 1 mL/5 x 10⁶ cells). This mixture was incubated for 60 minutes in a gently shaking 36°C water bath. The incubation was terminated by the addition of 30 mL ice-cold buffer B without albumin, followed by centrifugation at 800 g for ten minutes. The cells were washed three times with this buffer; clumps of cells that could not be resuspended were discarded (accounting for ~25% of the cells). The cells were finally suspended at 2 x 10⁶/mL in buffer B containing Ca²⁺ (1.53 mmol/L) but no albumin.

Nitrogen cationization and fractionation on Percoll gradients. ³²P-Labeled neutrophils were disrupted by nitrogen cavitation and fractionated on a discontinuous two-step Percoll gradient by a modification of the method of Borregaard et al. except that the cells were not treated with DFP. Membranes were harvested from the Percoll gradient as previously described and used immediately, either as a particulate preparation (2 x 10⁶ cell equivalents/mL in relaxation buffer) or after solubilization in deoxycholate according to Glass et al. ³²P Levels in these membranes amounted to 10,400 ± 1,800 cpm/10⁶ cell equivalents. Cytosol was obtained from similarly fractionated neutrophils, except that the cells had not been labeled with ³²P and had been treated with DFP before disruption.

O₂⁻ production by NADPH oxidase. Superoxide production was determined as previously described, using the continuous assay in which superoxide dismutase-inhibitable cytochrome c reduction was followed with time in a dual-beam spectrophotometer. Reaction mixtures contained 10⁶ cell equivalents of cytosol [251 ± 42 µg protein (n = 7)] and 8 x 10⁶ cell equivalents of membrane [25.6 ± 5.8 SD µg protein for membrane particles (n = 3) and 20.7 ± 5.9 µg protein for deoxycholate-solubilized membranes (n = 28)] in a total volume of 0.75 mL at 25°C. After baseline O₂⁻ production was measured, the NADPH oxidase in the assay mixture was activated by adding an optimum concentration of SDS (90 µmol/L for particles, 40 µmol/L for solubilized material). The rate of O₂⁻ production by the activated enzyme was followed at 550 nm.

Determination of phospholipid changes accompanying oxidative activation. Reaction mixtures were identical to those used for O₂⁻ measurements, except that the total volume was 1.5 mL, and cytochrome c, superoxide dismutase, and NADPH (and where indicated in Tables 1-3, other constituents) were omitted from the reaction mixture. Reactions were initiated by addition of ³²P-labeled membranes (either in particulate or solubilized form) with or without SDS. Incubations were conducted for the times indicated. The reactions were then terminated by addition of 5 mL chloroform/methanol (1/2, vol/vol) containing the antioxidant BHT (0.63 mg/mL) and authentic phosphoinositides (Sigma, 100 µg/mL). Phospholipids were extracted essentially according to Schach. Another 3.5 mL chloroform and 3.5 mL 2.4 N HC1 were added to the chloroform/methanol extract to generate aqueous and organic phases. The organic (lower) phase was removed, and the upper phase washed three times with 1 mL chloroform. The combined lower phases were then washed once with 1 mL methanol/1 N HC1 (1/1, vol/vol). The lower phase was removed, taken to dryness under nitrogen, and resuspended in 100 µL chloroform/methanol (2/1, vol/vol) for spotting on oxalate-impregnated TLC plates (Merck, silica gel 60 F254). The plates were washed once in 1 mL methanol/1 N HC1 (1/1, vol/vol) and three times with 1 mL chloroform.

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**Table 1. Effect of SDS on Activity of Respiratory Burst Oxidase and Content of Radioactive Phospholipids in Membrane Particles From ³²P-Labeled Human Neutrophils**

<table>
<thead>
<tr>
<th>Time of Incubation (s)</th>
<th>PIP (%)</th>
<th>PIP (%)</th>
<th>PI (%)</th>
<th>PC (%)</th>
<th>PA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 180</td>
<td>1.168 ± 73</td>
<td>1.425 ± 81</td>
<td>319 ± 4</td>
<td>451 ± 7</td>
<td>381 ± 4</td>
</tr>
<tr>
<td>15</td>
<td>1.199 ± 24</td>
<td>1.527 ± 7</td>
<td>346 ± 18</td>
<td>473 ± 27</td>
<td>380 ± 24</td>
</tr>
<tr>
<td>30</td>
<td>1.120 ± 53</td>
<td>1.504 ± 21</td>
<td>327 ± 5</td>
<td>445 ± 4</td>
<td>372 ± 5</td>
</tr>
<tr>
<td>60</td>
<td>1.134 ± 6</td>
<td>1.558 ± 79</td>
<td>343 ± 19</td>
<td>445 ± 19</td>
<td>374 ± 14</td>
</tr>
<tr>
<td>180</td>
<td>1.053 ± 92</td>
<td>1.498 ± 80</td>
<td>336 ± 14</td>
<td>449 ± 12</td>
<td>381 ± 5</td>
</tr>
</tbody>
</table>

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**Table 2. Failure of Inositol 1,4,5-Triphosphate and Activators of Protein Kinase C to Elicit O₂⁻ Production by Membrane Particles From Resting Neutrophils**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Ca²⁺ plus ATP</th>
<th>O₂⁻ Production (mol O₂⁻/min/10⁶ Cell eq Membranes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS (80–100 µmol/L)</td>
<td>106.4</td>
<td>48.8</td>
</tr>
<tr>
<td>Inositol triphosphate</td>
<td>+</td>
<td>44.2</td>
</tr>
<tr>
<td>10 µmol/L</td>
<td>+</td>
<td>+ *</td>
</tr>
<tr>
<td>1 µmol/L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PMA (0.2 µg/mL)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dioctanoylglycerol</td>
<td>10 µmol/L</td>
<td>+</td>
</tr>
<tr>
<td>1 µmol/L</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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O₂⁻ production was measured as described in the Materials and Methods section, except that the mixture of cytosol and membrane particles was incubated with the stimulus for 3.5 minutes at 25 °C before NADPH was added. The quantity of DMSO in the assay mixtures never exceeded 2 µL, a volume that had no effect on oxidative activation in the cell-free system. Where included, Ca²⁺ and ATP were present at final concentrations of 0.19 and 0.5 mmol/L, respectively.

*Below the limit of detection.
oxidase activation and lipid breakdown.

 Results and Discussion

Several groups of investigators have shown that certain anionic detergents, including cis-unsaturated fatty acids and SDS, are able to activate the respiratory burst oxidase in homogenates of resting neutrophils.10–14,17 We used this activating system to examine the relationship between oxidase activation and the catabolism of endogenous phosphoinositides. Lipids were labeled by incubating the neutrophils with $^{32}$P prior to homogenization. More than 70% of the radioactivity taken up by membrane lipids during this incubation appeared in PIP and PIP$_2$ (Table 1). Membranes were then isolated from the labeled cells for measurements of oxidase activation and lipid breakdown.

Initial experiments were made using cytosol plus membrane particles, with SDS as the activating detergent. As previously observed, SDS in the presence of cytosol was able to activate the oxidase in the neutrophil membrane, giving rise to $O_2^-$ production on addition of NADPH to the assay mixture. Labeled membrane phosphoinositides, however, did not fall in response to SDS at any point during the first three minutes of incubation under conditions that led to large-scale activation of the oxidase (Table 1; in this experiment, $O_2^-$ production had reached its maximum of 61 nmol/min/10$^7$ cell equivalents of membrane by three minutes after addition of the activating agent). These findings show that in this system the respiratory burst oxidase can be activated without phosphoinositide breakdown.

Conversely, the respiratory burst oxidase was not activated by either of the two products of PIP$_2$ breakdown: Diacylglycerol or inositol 1,4,5-trisphosphate (Table 2). No $O_2^-$ production occurred in response to addition of either of these secondary messengers to a system containing cytosol and membrane particles from human neutrophils. Similarly, PMA, which activates protein kinase C by serving as an analog of diacylglycerol,4 did not stimulate $O_2^-$ and membrane production occurred in response to addition of either of the two products of PIP$_2$ breakdown: Diacylglycerol or inositol 1,4,5-trisphosphate (Table 2). No $O_2^-$ production occurred in response to addition of either of these secondary messengers to a system containing cytosol and membrane particles from human neutrophils. Similarly, PMA, which activates protein kinase C by serving as an analog of diacylglycerol,4 did not stimulate $O_2^-$ production by this system. These findings provide further evidence that

Table 3. Effect of SDS on Activity of Respiratory Burst Oxidase and Content of Radioactive Phospholipids in Deoxycholate Extract of Membranes From $^{32}$P-Labeled Human Neutrophils

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>O$_2^-$ Production (nmol/min/mg protein)</th>
<th>Phospholipid (P$eta$, cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles, no SDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 s</td>
<td>0$^*$</td>
<td>2,770 2,331 417 1.31</td>
</tr>
<tr>
<td>3 min</td>
<td>0$^*$</td>
<td>2,506 2,181 406 1.31</td>
</tr>
<tr>
<td>Extract, no SDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 s</td>
<td>0$^*$</td>
<td>50 1,489 324 0.034</td>
</tr>
<tr>
<td>3 min</td>
<td>0$^*$</td>
<td>56 1,948 413 0.029</td>
</tr>
<tr>
<td>Extract, SDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 s</td>
<td>286</td>
<td>55 1,999 346 0.028</td>
</tr>
<tr>
<td>3 min</td>
<td>933</td>
<td>38 1,754 315 0.022</td>
</tr>
</tbody>
</table>

Reaction times were measured from the time membrane preparations were added to each reaction mixture. SDS was added at the same time as the membrane extract. The rates of O$_2^-$ production at 15 seconds and three minutes were calculated from the visually estimated tangents to the reaction progress curves. Results shown are representative of two experiments, each using neutrophils from a different donor.

*Based on data from other publications.8,14,17

Silica Gel 60 without fluorescent indicator. The plates were then developed in one dimension in a solvent system consisting of chloroform/acetone/methanol/acetic acid/water (80/30/26/24/14, vol/vol/vol/vol/vol).

Oxalate impregnation of TLC plates. Precoated plates (Silica gel 60, 20 x 20 cm, 250 µm thick, E Merck, Darmstadt, FRG) were impregnated with potassium oxalate by development for six hours with a solvent system containing 1.2% potassium oxalate in methanol/water (3/2, vol/vol). The plates were removed from the tanks and allowed to air dry. Plates were activated for 15 minutes at 110°C before spotting. The orientation of the plates during oxalate impregnation was retained during phospholipid chromatography.

Detection of radioactivity on TLC plates. Radioactive spots were detected by means of autoradiography (Kodak X-OMAT x-ray film) and by the AMBIS $\beta$-scanning system. Spots were quantitated using the AMBIS system after subtraction of machine background accumulated over a ten-hour scan. Spots were also quantitated using the conventional technique of scraping and counting in a Beckman LS 3801 liquid scintillation counter; cpm measured using the AMBIS system for $^{32}$P-labeled samples were ~20% of those obtained through scraping and counting. The two methods yielded comparable results (A.E. Traynor, Technical Note, AMBIS).
phosphoinositide breakdown is not involved in the activation of the respiratory burst oxidase in the cell-free system.

The results with PIP₂ were supported in experiments using a recently developed oxidase-activating system in which the particulate fraction was replaced by a fully soluble deoxycholate membrane extract whose activity remained in solution after a one-hour spin at 435,000 g. During the course of this extraction procedure, most of the labeled PIP₂ in the solubilized preparation (ca. 98%; Table 3) was lost, possibly due to the activation of a phospholipase C by the solubilizing detergent (Fig 1). (The absence of PIP₂ from the solubilized preparation did not represent a failure to extract this phospholipid from the membrane particles because the residual insoluble material was also nearly devoid of this substance.) Deoxycholate is known to stimulate phosphoinositide-specific phospholipase C from some sources.18 O₂⁻ Production was not detected when either NADPH alone or NADPH plus cytosol was added to the extract, indicating that the breakdown of PIP₂ does not itself activate the respiratory burst oxidase (Table 3). Just as with the unextracted membrane, however, the oxidase in the solubilized preparation was fully activated by addition of cytosol and SDS plus NADPH. In addition, oxidase activation occurred without any further change in the already greatly diminished content of labeled PIP₂ in the solubilized extract (Table 3).

Apart from activators of protein kinase C, most agents that stimulate human neutrophils activate both the respiratory burst oxidase and the phosphoinositide-catabolizing system of the cell.19-22 In the present studies, we were able to dissociate phosphoinositide catabolism from oxidase activation in an in vitro system by showing both that the respiratory burst oxidase can be activated without any change in the levels of labeled phosphoinositides and, conversely, that PIP₂ can be almost completely broken down without any detectable oxidase activation. These findings suggest that detergent-mediated activation of the respiratory burst oxidase in neutrophil homogenates takes place by a mechanism independent of the phosphoinositide-dependent signal-transducing system. Activation of the oxidase by detergents also appears to be independent of protein kinase C, since it does not require ATP.12 Nevertheless, detergent-mediated oxidase activation in the cell-free system appears to be closely related to oxidase activation by other stimuli in intact neutrophils, since inherited disorders that abolish oxidase activity in intact neutrophils (ie, the various types of chronic granulomatous disease) also abolish detergent-mediated oxidase activation in the cell-free system.8,12,16,23 For this reason, we believe that detergents stimulate oxidase activation through the normal signal transduction pathway. Because, however, detergent-mediated activation occurs without the participation of either phosphoinositides (this study) or protein kinase C,12 we conclude that, at least in the cell-free system, detergents activate this pathway at a relatively distal point, bypassing the molecular systems needed to propagate signals generated by more conventional agonists such as N-formylated oligopeptides and protein kinase C activators.

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Respiratory burst oxidase activation can be dissociated from phosphatidylinositol bisphosphate degradation in a cell-free system from human neutrophils

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