

# Cell-free Activation of the Respiratory Burst Oxidase by Protein Kinase C

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**ABSTRACT:** In intact neutrophils, phorbol ester treatment activates the respiratory burst oxidase, the enzyme responsible for O<sub>2</sub>- production by phagocytes. This effect is thought to be dependent on protein kinase C and on the phosphorylation of p47<sup>phox</sup>. In this paper, we report that protein kinase C activates the respiratory burst oxidase in a cell-free system consisting of isolated neutrophil cytosol and membrane. Oxidase activation required a highly active protein kinase C, recombinant p47<sup>phox</sup> and ATP, and was inhibited by the protein kinase C inhibitors H-7 and GF-109203X. Partial depletion of cytosolic ATP by dialysis reduced oxidase activation by over 50%. In contrast, neither protein kinase C inhibitors nor ATP depletion affected oxidase activation by SDS. These findings strongly suggest that in the cell-free system, the oxidase can be activated by the phosphorylation of p47<sup>phox</sup>.

**Keywords:** neutrophils, respiratory burst oxidase, protein phosphorylation, protein kinase C

## INTRODUCTION

Professional phagocytes and B cells possess an enzyme complex catalyzing the one electron reduction of molecular oxygen to superoxide (1-4). This enzyme, known as the respiratory burst oxidase, consists of a membrane-bound flavocytochrome known as cytochrome b<sub>558</sub> (5) and cytosolic components including p47<sup>phox</sup>, p67<sup>phox</sup> and the low molecular weight guanine nucleotide binding protein Rac2 (3,6,7). In the resting cell the enzyme is dormant, but it is brought to life by various stimuli that interact with membrane receptors [e.g., FMLP (N-formylmethionylleucyl-phenylalanine (8))] or activate intracellular kinases [e.g., PMA (phorbol myristate acetate) (9)]. It is likely that

in intact cells, protein phosphorylation regulates oxidase activation via p47<sup>phox</sup>, which is phosphorylated on several serines during oxidase activation (10-12). In addition to the whole cell activation, the oxidase can be activated in a cell-free system consisting of cytosol and membrane from resting cells and detergents like arachidonic acid and SDS (13-16). The cell-free system, however, shows differences from the physiological system since the former does not require protein phosphorylation for activation. In the present study we show that protein kinase C was able to activate the oxidase in a cell-free system. In addition to membranes and cytosol, activation by phosphorylation was absolutely dependent on added p47<sup>phox</sup> and protein kinase C.

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## MATERIALS AND METHODS

### Materials

Rat brain protein kinase C (86.8 U/ml; 2480 U/mg protein), horseradish peroxidase, H-7 and GF-109203X were from Calbiochem (La Jolla, CA). Superoxide dismutase (bovine erythrocyte), catalase, luminol, GTP $\gamma$ S, phosphatidylserine and diacylglycerol were purchased from Sigma (St. Louis).

### Isolation and Fractionation of Neutrophils

Neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrated blood. The neutrophils were suspended at a concentration of  $10^8$  cells/ml in a modified relaxation buffer (0.1 M KCl, 3 mM NaCl, 3.5 mM MgCl $_2$ , 10 mM PIPES buffer pH 7.3), and plasma membrane and cytosol were prepared by nitrogen cavitation and centrifugation through a Percoll gradient by the method of Borregaard (17). Both cytosol and membrane were divided into aliquots and stored at  $-70^\circ\text{C}$  until use. Where indicated, cytosol was dialyzed for 18 hrs against 3 changes of relaxation buffer and then treated with charcoal, to reduce the levels of endogenous nucleotides (18).

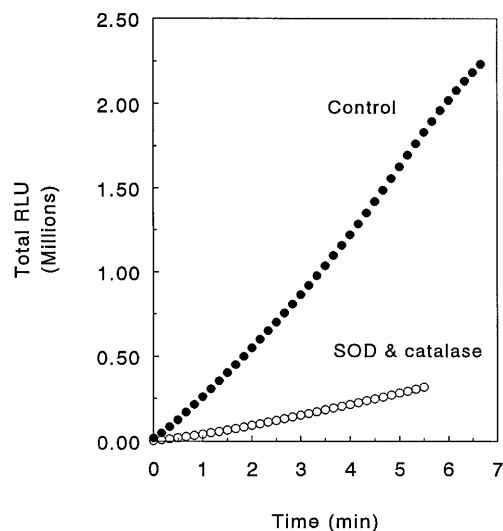
### Recombinant p47<sup>phox</sup>

Recombinant p47<sup>phox</sup> was obtained as a glutathione S-transferase fusion protein as previously described (19). Before use, excess glutathione was removed from the solution of purified recombinant protein by dialysis against relaxation buffer.

### Cell-Free Activation of the Respiratory Burst Oxidase

The activation mixture contained  $2.5 \times 10^7$  cell eq cytosol,  $10^7$  cell eq membranes, 0.5 U protein kinase C, 5  $\mu\text{g}$  of recombinant p47<sup>phox</sup>, 25  $\mu\text{g}$  phosphatidylserine, 2.5  $\mu\text{g}$  diacylglycerol, 0.5

mM CaCl $_2$ , 50  $\mu\text{M}$  GTP $\gamma$ S and ATP at 0.1 mM unless otherwise indicated, in 0.35 ml (final volume) relaxation buffer. Phosphatidylserine and diacylglycerol were added as mixed micelles that were prepared by dissolving the two lipids in chloroform, removing the chloroform under a stream of nitrogen and then sonicating the dried lipids for 2 min at room temperature in 20 mM Tris $\cdot$ Cl pH 7.4. After incubating for 5 min at  $37^\circ$ , oxidase activity was assayed by adding 18  $\mu\text{g}$  horseradish peroxidase, 10  $\mu\text{M}$  luminol and 0.16 mM NADPH, measuring chemiluminescence at room temperature in a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego) at successive 10 sec intervals. Activation with SDS was measured in the same way, except that in the activation mixture, protein kinase C and its activators (phosphatidylserine, diacylglycerol and CaCl $_2$ ) were replaced with 90  $\mu\text{M}$  SDS.



**Figure 1. Activation of the respiratory burst oxidase in a cell-free system by protein kinase C.** The experiments were carried out as described in Materials and Methods. Concentrations of superoxide dismutase and catalase were 20  $\mu\text{g}/\text{ml}$  and 10  $\mu\text{g}/\text{ml}$  respectively. These results are representative of 9 experiments. SOD = superoxide dismutase; RLU = relative luminescence units.

## RESULTS

In a system containing resting neutrophil cytosol and membranes plus additional p47<sup>phox</sup> (added as the recombinant protein), the

respiratory burst oxidase could be activated by protein kinase C. Fig. 1 shows that in a mixture containing resting neutrophil components, protein kinase C, its activators (phosphatidylserine, diacylglycerol and calcium), and extra  $p47^{phox}$ , oxidants were produced as detected by light emission. Luminescence was decreased to 20% of control by superoxide dismutase plus catalase; a similar decrease was obtained with superoxide dismutase alone (data not shown). These results indicate that in the system employed, the respiratory burst oxidase was activated to produce  $O_2^-$ .

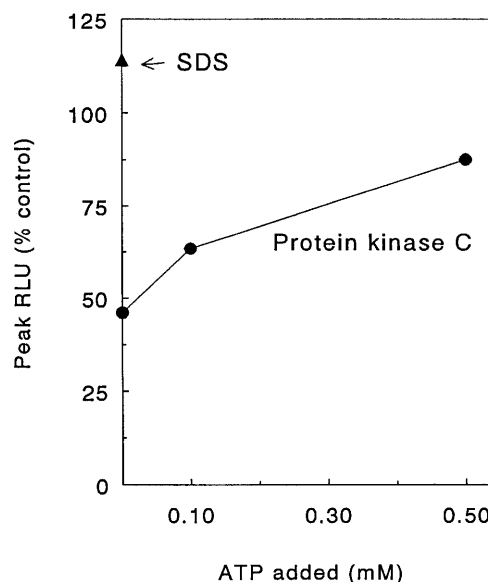
**Table I. Requirements for activation of cell-free respiratory burst oxidase by protein kinase C.** Experiments were carried out as described in Materials and Methods, with omissions as noted. Oxidase activity was assayed at room temperature in the luminometer. Peak activities for each set of conditions are expressed as % peak control activity  $\pm$  1 SE. N refers to the number of experiments performed under each set of conditions. Peak control activity averaged  $33400 \pm 6700$  SE relative luminescence units.

| Reaction mixture      | Peak activity % of control | N |
|-----------------------|----------------------------|---|
| Complete              | 100                        | 4 |
| - NADPH               | $0.15 \pm 0.003$           | 2 |
| - $p47^{phox}$        | $4.2 \pm 1.6$              | 4 |
| - Protein kinase C    | $23.4 \pm 6.7$             | 4 |
| + 2x protein kinase C | 167.6                      | 1 |
| - Cytosol             | $20.6 \pm 0.7$             | 2 |
| - Membranes           | $7.4 \pm 5.0$              | 2 |

Oxidase activation was dependent on each of the components in the mixture (Table I).  $O_2^-$  production was greatly diminished by the omission of protein kinase C, cytosol or membranes. Conversely,  $O_2^-$  production rose when the amount of protein kinase C in the assay mixture was increased. Residual  $O_2^-$  production in some of the omission experiments is probably attributable to the activity of the small amount of protein kinase C in the cytosol, or to residual cytosol in the membrane preparation.

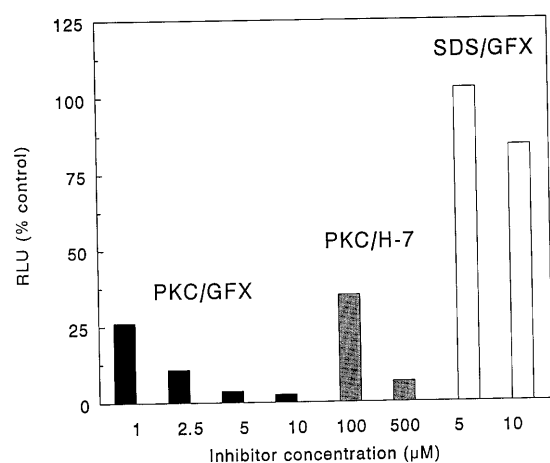
The dependence of oxidase activation on ATP was tested using cytosol that had been dialyzed overnight to reduce the levels of nucleotides. Overnight dialysis did not eliminate

ATP completely (18,20), but reduced its level sufficiently to decrease luminescence to <50% of control when ATP was not added to the system (Fig. 2). (Some ATP might also have been introduced into the reaction mixture with the [undialyzed] membrane preparation.) The addition of ATP to the system returned  $O_2^-$  production to near control levels in a concentration-dependent manner.



**Figure 2. Dependence of oxidase activation on ATP.** The experiments were carried out as described in Materials and Methods, using dialyzed cytosol and adding ATP at the concentrations indicated. Results are presented as % peak control activities. Peak activities for the protein kinase C and SDS controls were 28000 and 242300 RLU (relative luminescence units)/sec.

Protein kinase C inhibitors were used to confirm the role of protein kinase C in oxidase activation. The two inhibitors H-7 and GF-109203X were used in these experiments, the former inhibiting several kinases (21) and the latter more specific for protein kinase C (22). Both of these compounds decreased light emission in the protein kinase C-activation system by more than 90% (Fig. 3). Light emission in response to SDS, however, was unaffected, indicating that neither inhibitor acts directly on the oxidase or interferes with the assay.



**Figure 3. Effect of protein kinase C inhibitors on protein kinase C-mediated oxidase activation in a cell-free system.** The experiments were carried out as described in Materials and Methods. Peak activities for the protein kinase C and SDS controls were 27600 and 134900 RLU (relative luminescence units)/sec, respectively. Inhibitors and activators are indicated in the figure. Abbreviations: PKC, protein kinase C; GFX, GF-109203X.

## DISCUSSION

The activation of human neutrophils by phorbol myristate acetate, a protein kinase C activator, leads to  $O_2^-$  production by these cells. The oxidase subunit  $p47^{phox}$ , known to be phosphorylated during oxidase activation, is a good substrate for protein kinase C *in vitro* (23), while staurosporine, a potent inhibitor of protein kinase C and other protein kinases, prevents both  $O_2^-$  production and the phosphorylation of  $p47^{phox}$  by phorbol-treated neutrophils (24,25). We recently showed that during neutrophil activation, several serines in the C-terminal quarter of  $p47^{phox}$  are phosphorylated (12), and that at least one of these phosphorylation targets, namely S379, is required for oxidase activity (26). In this report we furnish direct evidence that protein kinase C is able to activate the respiratory burst oxidase. Protein kinase C-mediated activation appears to require protein phosphorylation, but is independent of unsaturated fatty acids, alkyl sulfates and sulfonates, and phosphatidic acid, the anionic detergents that had been shown in the past to

activate the cell-free oxidase (13,14,27-29). It thus provides different information about physiological oxidase activation than the detergent-based systems hitherto employed.

Recently McPhail et al (20) described a cell-free oxidase activating system that was regulated by ATP, but this system employed as the activating agent phosphatidic acid, an anionic detergent that can also activate the cell-free oxidase in the absence of protein phosphorylation (28,29). To our knowledge the only previous report describing findings similar to ours was that by Tauber's group (30). In that study it was shown that protein kinase C was able to elicit limited but real oxidase activity from a cell-free system containing cytosol and membranes from resting neutrophils. Our results confirm most of their findings, though we could not reproduce their observation that protein kinase C alone could replace neutrophil cytosol, a discrepancy that suggests that their neutrophil membrane preparation may not have been completely free of cytosol. In any case, the data presented here suggest that the limiting component in both the present study and their earlier study was phosphorylated  $p47^{phox}$ .

We have shown that the activation of the respiratory burst oxidase by SDS creates a membrane binding site on one or more of the oxidase components. We believe that the phosphorylation of  $p47^{phox}$  has a similar effect. Specifically, we postulate that by neutralizing the very strong positive charge of the arginine- and lysine-rich C-terminal tail of  $p47^{phox}$ , phosphorylation allows the displacement of that tail from its location in the unphosphorylated protein, exposing sites to which other polypeptides can bind to assemble the active oxidase (31). In this connection, evidence has been presented that a  $p47^{phox}$  SH3 domain that in resting cells is associated with a proline-rich domain on the same molecule changes partners during activation to associate with a proline-rich domain on the  $\alpha$  subunit of the membrane-associated  $\alpha$  subunit of cytochrome  $b_{558}$ . It is possible that this change in the configuration of  $p47^{phox}$  is mediated by the phosphorylation of its

tail, and that a similar change can be accomplished through an interaction between p47<sup>phox</sup> and the anionic detergents (SDS, arachidonic acid and phosphatidic acid) that have been shown to activate the oxidase in the cell-free system.

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#### REFERENCES

- Babior BM: Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* 298, 659-668, 1978.
- Malech HL, Gallin JI: Current concepts: Immunology. Neutrophils in human disease. *N Engl J Med* 317, 687-694, 1987.
- Chanock SJ, El Benna J, Smith RM, Babior BM: The respiratory burst oxidase. *J Biol Chem* 269, 24519-24522, 1994.
- Maly FE, Cross AR, Jones OT, Wolf-Vorbeck G, Walker C, Dahinden CA, de Weck AL: The superoxide generating system of B cell lines. Structural homology with the phagocytic oxidase and triggering via surface Ig. *J Immunol* 140, 2334-2339, 1988.
- Segal AW: Cytochrome b-245 and its involvement in the molecular pathology of chronic granulomatous disease. In: Curnutte JT, ed., *Hematology/Oncology Clinics of North America. Phagocytic Defects II*, Vol 2, Philadelphia, W.B. Saunders, 1988, p. 213.
- Nauseef WM: Cytosolic oxidase factors in the NADPH-dependent oxidase of human neutrophils. *Eur J Haematol* 51, 301-308, 1993.
- Lomax KJ, Malech HL, Gallin JI: The molecular biology of selected phagocyte defects. *Blood Rev* 3, 94-104, 1989.
- Jesaitis AJ, Tolley JO, Allen RA: Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. *J Biol Chem* 261, 13662-13669, 1986.
- Wolfson M, McPhail LC, Nasrallah VN, Snyderman R: Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: Potential role in the activation of the respiratory burst enzyme. *J Immunol* 135, 2057-2062, 1985.
- Hayakawa T, Suzuki K, Suzuki S, Andrews PC, Babior BM: A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils. *J Biol Chem* 261, 9109-9115, 1986.
- El Benna J, Ruedi JM, Babior BM: Cytosolic guanine nucleotide-binding protein Rac2 operates *in vivo* as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47<sup>phox</sup> and p67<sup>phox</sup> to the submembranous actin cyto-skeleton during oxidase activation. *J Biol Chem* 269, 6729-6734, 1994.
- El Benna J, Faust LP, Babior BM: The phosphorylation of the respiratory burst oxidase component p47<sup>phox</sup> during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. *J Biol Chem* 269, 23431-23436, 1994.
- Bromberg Y, Pick E: Unsaturated fatty acids stimulate NADPH-dependent superoxide production by cell-free system derived from macrophages. *Cell Immunol* 88, 213-221, 1984.
- Pick E, Bromberg Y, Sphungin S, Gadba R: Activation of the superoxide forming NADPH oxidase in a cell-free system by sodium dodecyl sulfate. Characterization of the membrane-associated component. *J Biol Chem* 262, 16476-16483, 1987.
- Curnutte JT: Activation of human neutrophil nicotinamide adenine dinucleotide phosphate, reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic acid in a cell-free system. *J Clin Invest* 75, 1740-1743, 1985.
- McPhail LC, Shirley PS, Clayton CC, Snyderman R: Activation of the respiratory burst enzyme from human neutrophils in a cell-free system: Evidence for a soluble cofactor. *J Clin Invest* 75, 1735-1739, 1985.
- Borregaard N, Heiple JM, Simons ER, Clark RA: Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* 97, 52-61, 1983.
- Peveri P, Heyworth PG, Curnutte JT: Absolute requirement for GTP in activation of human neutrophil NADPH oxidase in a cell-free system: Role of ATP in regenerating GTP. *Proc Natl Acad Sci USA* 89, 2494-2498, 1992.
- Park J-W, El Benna J, Scott KE, Christensen BL, Chanock SJ, Babior BM: Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytosol. *Biochemistry* 33, 2907-2911, 1994.
- McPhail LC, Qualliotine-Mann D, Waite KA: Cell-free activation of neutrophil NADPH oxidase by a phosphatidic acid-regulated protein kinase. *Proc Natl Acad Sci* 92, 7931-7935, 1995.
- Hidaka H, Inagaki M, Kawamoto S, Sasaki Y: Isoquinoline sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* 23, 5036, 1984.
- Toullec D, Pranetti P, Coste M, Bellevergue P, Giraud-Perret T, Ajakawe M, Baudet V, Boissin P,

- Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky H: The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of PKC. *J Biol Chem* 266, 15771, 1991.
23. Kramer IJM, Verhoeven AJ, van der Bend RL, Weening RS, Roos D: Purified protein kinase C phosphorylates a 47 kDa protein in control neutrophil cytoplasts but not in neutrophil cytoplasts from patients with the autosomal form of chronic granulomatous disease. *J Biol Chem* 263, 2352-2357, 1988.
  24. Combadiere C, El Benna J, Pedruzzi E, Hakim J, Perianin A: Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine. *Blood* 82, 2890-2898, 1993.
  25. Nauseef WM, Volpp BD, McCormick S, Leidal KG, Clark RA: Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J Biol Chem* 266, 5911-5917, 1991.
  26. Faust LP, El Benna J, Babior BM, Chanock SJ: The phosphorylation targets of p47phox, a subunit of the respiratory burst oxidase. Functions of the individual target serines as evaluated by site-directed mutagenesis. *J Clin Invest* 96, 1499-1505, 1995.
  27. Badwey JA, Curnutte JT, Robinson JM, Berde CB, Karnovsky MJ, Karnovsky ML: Effects of free fatty acids on release of superoxide and on change of shape by human neutrophils: Reversibility by albumin. *J Biol Chem* 259, 7870-7877, 1984.
  28. Bellavite P, Corso F, Dusi S, Grzeskowiak M, Della-Bianca V, Rossi F: Activation of NADPH-dependent superoxide production in plasma membrane extracts of pig neutrophils by phosphatidic acid. *J Biol Chem* 263, 8210-8214, 1988.
  29. Qualliotine-Mann D, Agwu DE, Ellenburg MD, McCall CE, McPhail LC: Phosphatidic acid and diacylglycerol synergize in a cell-free system for activation of NADPH oxidase from human neutrophils. *J Biol Chem* 268, 23843-23849, 1993.
  30. Cox JA, Jeng AY, Sharkey NA, Blumberg PM, Tauber AI: Activation of the human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C. *J Clin Invest* 76, 1932, 1985.
  31. Park J-W, Ma M, Ruedi JM, Smith RM, Babior BM: The cytosolic components of the respiratory burst oxidase exist as a Mr 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. *J Biol Chem* 267, 17327-17332, 1992.