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MINI-REVIEW

Subcellular Localization and Dynamics of Components of the Respiratory Burst Oxidase

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Abstract

Membrane and cytosolic factors cooperate to generate NADPH-oxidase. The study of the syndrome of NADPH-oxidase deficiencies, chronic granulomatous disease, has enabled the identification of two membrane factors: a flavin adenine dinucleotide flavoprotein and a *b* cytochrome. The nature of the cytosolic components is still unknown, but a 47-kD protein, whose phosphorylation occurs in parallel with the generation of a respiratory burst in intact cells, seems to be one of the cytosolic factors. The subcellular localization of the membrane-bound NADPH-oxidase components has been studied in neutrophils: In unstimulated cells, only a minute fraction of the NADPH-oxidase components is localized in the plasma membrane, whereas ~80% is localized in the membrane of the specific granules and the majority of the rest is in a newly described membrane-bound compartment, the secretory granules, identified by latent alkaline phosphatase. During stimulation, these NADPH-oxidase components are translocated to the plasma membrane as a result of fusion of granule membrane with plasma membrane. Only the NADPH-oxidase components present in the plasma membrane are incorporated in the respiratory burst oxidase generated in intact cells.

Key Words: Cytochrome *b*; exocytosis; flavoprotein NADPH-oxidase; neutrophils; protein kinase C; secretory granules; specific granules.

Introduction

The extra respiration of phagocytosis was first reported by Baldridge and Gerard (1933) in their study on canine neutrophils. Sbarra and Karnovsky (1959) established the nonmitochondrial nature of this respiratory burst of phagocytosis, and Iyer and Quastel (1963) and Rossi and Zatti (1964)

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identified a nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase as the biochemical basis for the respiratory burst. This oxidase, absent from resting neutrophils and appearing within minutes after stimulation, has turned out to be very complex, and the term oxidase, which indicates that it is a simple enzyme, is hardly suitable to describe the multicomponent electron transport system that now materializes as NADPH-oxidase.

Identification of chronic granulomatous disease (CGD) as a disease entity characterized by a profound deficiency of microbicidal activity of phagocytes due to inability of the phagocytes to mount a respiratory burst (Holmes *et al.*, 1967; Tauber *et al.*, 1983; Curnutte and Babior, 1987) has been of paramount importance for identification of the role of the respiratory burst in host defense and tissue injury, and has been essential in establishing the nature of the respiratory burst oxidase as a superoxide generating NADPH oxidase (Curnutte and Babior, 1974). The study of CGD cells has also been invaluable for identification of the components that are involved in generating the NADPH oxidase.

Until 1984, the oxidase could only be studied in preparations obtained from stimulated phagocytes (neutrophils, monocytes, eosinophils, and macrophages). The observation (Heineman and Verkauteren, 1984; Bromberg and Pick, 1984, 1985; McPhail *et al.*, 1985; Curnutte, 1985) that an NADPH-oxidase, very similar to that observed from stimulated cells, can be generated by combining cytosol and membrane fractions from unstimulated cells in the presence of suitable activators has added greatly to the potential for studying the structure and mode of activation of NADPH-oxidase.

The Components of NADPH-oxidase

Three approaches have been taken to identify NADPH oxidase:

1) Characterization of NADPH-oxidase that is generated in intact cells after stimulation. This approach precludes identification of the initial steps involved in assembly of the oxidase, and carries the possible pitfall of the oxidase being altered during the isolation procedure.

2) The use of CGD cells as a gold standard. The reason for the use of CGD has been the argument that, since the CGD abnormality seems to involve only the respiratory burst, any abnormality associated with CGD must reflect an abnormality of the respiratory burst machinery. The two main drawbacks of this approach are the possibility of linking CGD defects to other defects not involving the respiratory burst, e.g., the McLeod type (Marsh *et al.*, 1975; Frey *et al.*, 1988), and the possibility that not all components necessary to constitute NADPH-oxidase are represented by corresponding defects, recognized as manifestations of CGD.

3) Characterization of the structures that participate in generation of NADPH-oxidase in subcellular fractions from unstimulated cells. The obvious weakness of this approach is the possibility that NADPH-oxidase generated in this so-called cell-free system may not entirely reflect NADPH-oxidase generated in the intact cell in response to stimulation.

These three approaches have been complementary in their contribution to the current understanding of NADPH-oxidase. Although the exact nature of NADPH-oxidase is not fully known, the following now stands as dogma:

1) *Respiratory burst oxidase in an NADPH-oxidase.* This has been established both for oxidase generated by activation of cells (Babior, 1983; Glass *et al.*, 1986) and oxidase generated in cell-free systems (Clark *et al.*, 1987, Curnutte *et al.*, 1987b; Babior *et al.*, 1988).

2) *One component of NADPH-oxidase is a unique low-potential cytochrome b* that is composed of two subunits—a 22-kD nonglycosylated polypeptide and a 91-kD glycosylated polypeptide—which are noncovalently linked to each other (Parkost *et al.*, 1987; Segal, 1987). Evidence for the participation of cytochrome *b* as part of NADPH-oxidase is both genetic (Segal *et al.*, 1983; Dinauer *et al.*, 1987; Teahan *et al.*, 1987) and functional (Segal and Jones, 1979; Borregaard *et al.*, 1982). This is further supported by the observed colocalization of cytochrome *b* and NADPH-oxidase activity in subcellular fractions used in the cell-free system (Pick *et al.*, 1987; Clark *et al.*, 1987; Bjerrum and Borregaard, 1988; Dotan *et al.*, 1988), and by the presence of cytochrome *b* in preparations of the NADPH-oxidase from stimulated cells (Markert *et al.*, 1985; Bellavite *et al.*, 1983; Green and Pratt, 1988) with two exceptions (Glass *et al.*, 1986; Kakinuma *et al.*, 1987).

3) *One component of NADPH-oxidase is a flavin adenine dinucleotide (FAD) flavoprotein.* The evidence for this is indirect and is based on the requirement for FAD for full activity of NADPH-oxidase preparations from activated cells (Babior and Kipnes, 1977; Light *et al.*, 1981) and of NADPH-oxidase preparations generated in cell-free systems (Bromberg and Pick, 1984, 1985) when some detergents are included during isolation of the oxidase. It has further been demonstrated by electron-spin resonance that electrons are added from NADPH to flavoproteins in the plasma membrane from stimulated cells, but not from unstimulated cells (Kakinuma *et al.*, 1986). All reports on purified NADPH-oxidase preparations from stimulated cells, with one exception (Bellavite *et al.*, 1986), have found FAD to be a copurifying factor. Some studies on CGD cells have demonstrated a deficiency of FAD associated with deficiency of cytochrome *b* (Cross *et al.*, 1982; Borregaard and Tauber, 1984; Gabig and Lefker, 1984; Ohno *et al.*, 1986; Bohler *et al.*, 1986) and in a single case also with cytochrome-*b*-positive CGD cells

(Gabig, 1983), but no consensus exists about the incidence of FAD deficiency in CGD cells or its association with cytochrome *b* deficiency.

4) *Expression of NADPH-oxidase activity in intact cells depends on phosphorylation of a 47-kD protein.* The evidence for this is mainly genetic: Phosphorylation of the 47-kD protein is deficient in neutrophils from autosomal CGD that have normal cytochrome *b* content (Segal *et al.*, 1986; Heyworth and Segal, 1986; Hayakawa *et al.*, 1986; Kramer *et al.*, 1988). Recent evidence (Kramer *et al.*, 1988; Caldwell *et al.*, 1988) indicates that the defective phosphorylation is due to absence or defects in the 47-kD protein and not due to malfunction of its kinase. No agreement exists about whether phosphorylation of the 47-kD protein is also essential for activation of NADPH-oxidase in the cell-free system (Clark *et al.*, 1987; Caldwell *et al.*, 1988; Babior *et al.*, 1988).

5) *NADPH-oxidase is assembled by combining soluble "cytosolic" components with membrane-bound components.* The evidence is based on complementation experiments using membrane and cytosol from normals and CGD patients (Curnutte, 1985; Curnutte *et al.*, 1987b, 1988). It may be argued that these *in vitro* studies are not necessarily reflections of *in vivo* activation, but the necessity for different components to interact in order to generate NADPH-oxidase *in vivo* is documented by the complementation experiments in which monocytes from different CGD subsets are fused and become able to generate a respiratory burst (Hamers *et al.*, 1984; Weening *et al.*, 1985). The nature of this interaction between membrane-bound components and cytosolic factors is not yet established. Activation may very likely occur through different pathways, depending on the stimulus that is used to activate the oxidase. Phosphorylation of the 47-kD protein by cytosolic factors seems to be involved as discussed below. Much effort is now directed at establishing the nature of these membrane and cytosolic factors. It should be noted that the so-called cytosolic factors have not been shown to be released from the cells by permeabilization, but are judged to be cytosolic simply by their distribution after centrifugation of subcellular fractions. Thus, the possibility of a loose membrane attachment cannot be excluded.

It is generally agreed that one of the membrane factors is cytochrome b_{-245}. It is not fully agreed as to whether the FAD component is membrane bound in the unactivated cell, although subcellular fractionation studies support this (Borregaard and Tauber, 1984). Recent studies on NADPH-oxidase preparations generated in cell-free systems indicate that the 47-kD protein is one of the cytosolic factors required for *in vitro* generation of NADPH oxidase (Bolscher *et al.*, 1988). Caldwell *et al.* (1988) reported that cytosol contains the factor(s) necessary for phosphorylation of the 47-kD protein, which, in their system, appears to be membrane bound. A unifying concept would be that the 47-kD protein is cytosolic, but anchors to the membrane when phosphorylated.

In addition to these concepts, some evidence has been presented that a ubiquinone may be part of NADPH-oxidase. The evidence is entirely indirect and is based on the identification of ubiquinone in phagocytic vacuoles, and a change in their oxidation state during activation of the neutrophil (Crawford and Schneider, 1982, 1983). Although others have confirmed the presence of ubiquinone in neutrophils (Cunningham *et al.*, 1982; Lee, 1986), Cross *et al.* (1983) found that ubiquinone was only associated with neutrophil fractions containing mitochondria, and was absent from fractions containing other NADPH-oxidase components. Lutter *et al.* (1984) found no ubiquinone in granule- and mitochondria-depleted cytoplasts that retain a significant capacity for generating a respiratory burst and thus contain NADPH-oxidase components sufficient to assemble a working NADPH-oxidase. It was suggested that contamination by platelets might have accounted for the presence of ubiquinone in the preparations of neutrophils reported by Schneider and coworkers.

It can be concluded that the following components are necessary to constitute NADPH-oxidase: cytochrome b_{-245}; a FAD flavoprotein, not yet identified, but probably identical to the FAD flavoprotein identified as part of the NADPH-oxidase purified by Glass *et al.* (1986); a 47-kD protein, the phosphorylation of which is necessary for induction of NADPH-oxidase activity; and at least one additional "cytosolic" factor, not yet identified. It should be kept in mind, however, that NADPH-oxidase has not been reconstituted by assembly of these components in their isolated form, and the order in which the individual components act to permit electron flow from NADPH to O_2 is not established. A model of NADPH-oxidase consistent with most findings is presented in Fig. 1. It should be stressed that this model

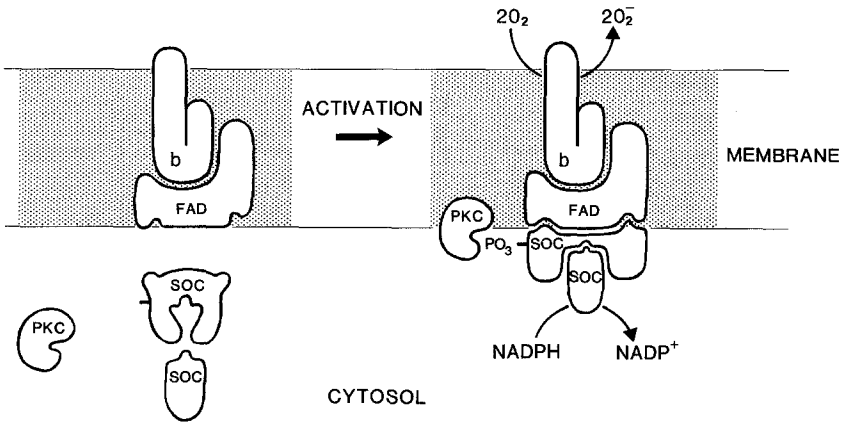


Fig. 1. Hypothetical model of NADPH-oxidase components in resting and stimulated cells: b, cyotochrome *b*; FAD, flavoprotein; PKC, protein kinase C; and SOC, soluble (cytosolic) oxidase components.

is hypothetical and, in particular, that the localization of the NADPH-binding site and of the 47-kD protein is not yet established.

Electron Flow Through NADPH-oxidase

Some studies have been conducted to define the route of electron flow through the components of NADPH-oxidase. Cross *et al.* (1981) established that cytochrome *b* can react with CO. This indicates that the cytochrome may deliver electrons directly to oxygen. Earlier calculations which concluded that the rate of reduction of cytochrome *b* in NADPH-oxidase preparations was much too slow to permit any role for cytochrome *b* in electron transport associated with the respiratory burst (Gabig *et al.*, 1982) have been overruled by the stop-flow experiments of Cross *et al.* (1985), who demonstrated that O_2 is necessary for proper reduction of cytochrome *b* by NADPH. Since cytochromes can only pass one electron at a time, cytochrome *b* cannot be expected to accept electrons directly from NADPH, which can only emit two electrons at a time. It is therefore assumed that electrons are emitted pairwise to the flavoprotein component of NADPH-oxidase and passed sequentially to the cytochrome *b* component, but no studies have demonstrated reduction of the flavoprotein components by NADPH when electron flow through the cytochrome *b* component is blocked. Thus, direct evidence for the route of electron flow through the components of NADPH-oxidase is lacking.

Subcellular Localization of the NADPH-oxidase

NADPH-oxidase in Activated Cells

It is important to make a distinction between the NADPH-oxidase generated in intact cells, and the NADPH-oxidase that may be generated in cell-free systems. The subcellular localization of NADPH-oxidase generated in intact cells has been visualized histochemically as the site of H_2O_2 production in intact stimulated cells (Badwey *et al.*, 1980; Ohno *et al.*, 1982), or functionally as the NADPH-dependent superoxide anion generation in subcellular fractions obtained by density-gradient centrifugation of homogenates of stimulated cells (Dewald *et al.*, 1979; Borregaard and Tauber, 1984). Both approaches have yielded results which demonstrate that the NADPH-oxidase generated by the cells is localized exclusively in the plasma membrane and phagosomal membrane.

Cytochrome b and FAD Flavoprotein

It has long been recognized that the specific granules of human neutrophils contain the majority of the cytochrome *b* component of NADPH-oxidase (Sloan *et al.*, 1981; Borregaard *et al.*, 1983; Parkost *et al.*, 1985; Ohno *et al.*, 1985). We found also that the major part of total cell FAD was associated withh specific granules and, in particular, that FAD flavoprotein of the specific granules was deficient in cytochrome-*b*-negative CGD patients, thus indicating that this flavoprotein is of relevant for NADPH-oxidase (Borregaard and Tauber, 1984). We further demonstrate that these NADPH-oxidase components translocate to the plasma membrane as a consequence of exocytosis of specific granules during stimulation (Borregaard *et al.*, 1983; Borregaard and Tauber, 1984 (Fig. 2). These observations generated the hypothesis that specific granules might serve as important stores of NADPH-oxidase components that may nourish plasma membrane NADPH-oxidase during activation. Some calculations have been performed which indicate that such traffic of NADPH-oxidase components is not necessary for full

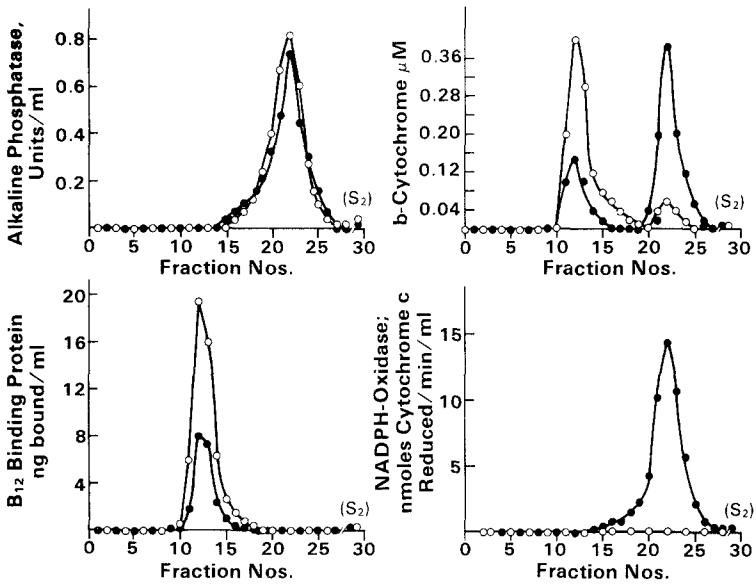


Fig. 2. Subcellular localization of NADPH-oxidase and cytochrome *b* in resting and PMA-stimulated neutrophils: 9×10^8 neutrophils were incubated with PMA 2 μg/ml, and then disrupted by nitrogen cavitation and fractionated on Percoll density gradients. Fractions of 1.2 ml each were collected and assayed for plasma membrane (alkaline phosphatase), specific granules (B_{12}-binding protein), cytochrome *b* content, and NADPH-oxidase activity. Note that NADPH-oxidase is measured without activators: (○) control cells and (●) activated cells. Reproduced from Borregaard and Tauber (1984).

NADPH-oxidase activity (Parkost *et al.*, 1985). Nonetheless, the hypothesis is consistent with the observation that stimuli which induce extensive exocytosis of specific granules like phorbol myristate acetate (PMA) also elicit huge respiratory burst activity, whereas stimuli which only cause little or no exocytosis of specific granules like formyl-methionyl-leucyl-phenylalanine (fMLP) only induce minimal respiratory burst activity.

It has, however, been an unresolved question as to whether the granule membrane is fully competent as a source of membrane-bound NADPH-oxidase components as discussed below.

It should be noted that gelatinase-containing granules have been claimed to contain cytochrome *b* (Mollinedo and Schneider, 1984). Immunogold labeling has demonstrated that gelatinase is colocalized with the specific granule marker lactoferrin (Hibbs *et al.*, 1986). We have found that the separation of peak genatinase activity from peak vitamine B_{12}-binding capacity (another marker for specific granules) on density gradients (Borregaard *et al.*, 1987) may be explained by destruction of gelatinase by proteases present in azurophil granules that contaminate the specific granules (Bjerrum and Borregaard, unpublished).

Dormant NADPH-oxidase

The possibility of activating NADPH-oxidase in cell-free systems has enabled ways to study the localization of components of NADPH-oxidase in unactivated cells, the dormant NADPH-oxidase. Cytosol which contains soluble NADPH-oxidase components is combined with various particulate fractions obtained by density-gradient centrifugation, and an activator, either arachidonate or sodium dodecyl sulfate (SDS) (Heineman and Verkauteren, 1984; Bromberg and Pick, 1985; Curnutte *et al.*, 1987a; Clark *et al.*, 1987; Bjerrum and Borregaard, 1988). In this way, the membrane-bound part of NADPH-oxidase may be quantitated in various subcellular membrane-bound compartments. Clark *et al.* (1987) and Bjerrum and Borregaard (1988) reported that the majority of the total membrane-bound NADPH-oxidase components are localized in the specific granules, and only a minor fraction is localized in the plasma membrane. In contrast, Curnutte *et al.* (1987a), using similar fractionation techniques, recently found that only the plasma membrane contains all membrane-bound factors necessary to constitute NADPH-oxidase. It has recently been demonstrated, however, that NADPH-oxidase is rapidly inactivated by proteases present in azurophil granules (Bjerrum and Borregaard, 1988). Azurophil granules are always present as contaminants in the specific granule peak fractions when isolated on Percoll density gradients (Borregaard *et al.*, 1983) as used by both Clark *et al.* and Curnutte *et al.* Thus, proteolytic destruction of NADPH

components in specific granules may explain the inability of Curnutte *et al.* to measure NADPH-oxidase activity in specific granule fractions.

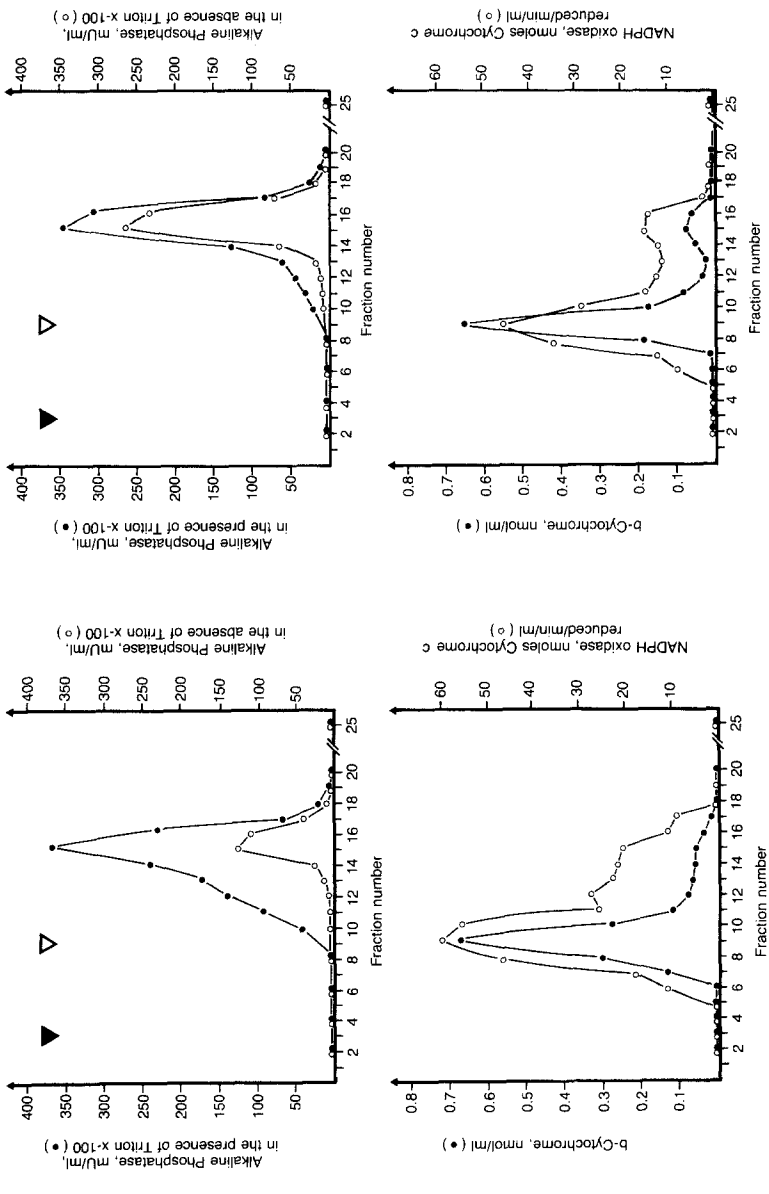
We recently identified a membrane-bound, easily mobilizable compartment in neutrophils (Borregaard *et al.*, 1987). This compartment is completely exocytosed in response to stimulation of intact cells by agents such as nanomolar concentrations of fMLP or leukotriene B_4 (LTB_4). This compartment, provisionally named the secretory granule, is identified by latent alkaline phosphatase and tetranectin (Borregaard *et al.*, 1988). The secretory granules are localized close to, but distinct from, the plasma membrane on Percoll density gradients and contain the majority of cytochrome *b* and other membrane-bound NADPH-oxidase components that we hitherto considered to be localized in the plasma membrane (Fig. 3). Stimulation by weak secretagogues preferentially mobilizes the secretory granules, whereas more potent secretagogues mobilize both granule compartments. This dual granule localization may thus explain the differences in the respiratory burst elicited by different stimuli.

Although some situations may exist in which the NADPH-oxidase of granules may be activated without exocytosis occurring (Dahlgren, 1987), it is the general view that only the NADPH-oxidase in the plasma membrane is activated after stimulation of cells as discussed above. It is very likely, although not proven, that the NADPH-oxidase components that are translocated to the plasma membrane from the specific and secretory granules constitute the major part of NADPH-oxidase in the plasma membrane of activated cells.

Control of Activation of NADPH-oxidase

As discussed above, NADPH-oxidase may be assembled in cell-free systems by combing an activator, like SDS, with cytosolic factors and membrane-bound factors present in specific granules, in secretory granules, and in plasma membranes. In the unactivated cell, the vast majority of membrane-bound factors (80%) are present in specific granules, 15% are located in secretory granules, and only 5% reside in the plasma membrane (Bjerrum and Borregaard, 1988). This shows that all components necessary to constitute an NADPH-oxidase are present in these intracellular stores. During exocytosis of granules, the membrane-bound part of the NADPH-oxidase present in the granule membrane is translocated to the plasma membrane. In this way, control of respiratory burst activity may be linked to control of exocytosis.

An obvious question then is what is the mechanism which ensures that only the NADPH-oxidase components present in the plasma membrane


Alkaline Phosphatase, mU/ml,
in the presence of Triton x-100 (•)
Alkaline Phosphatase, mU/ml,
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NADPH oxidase, nmoles Cytochrome c
reduced/min/ml (○)
Fraction number

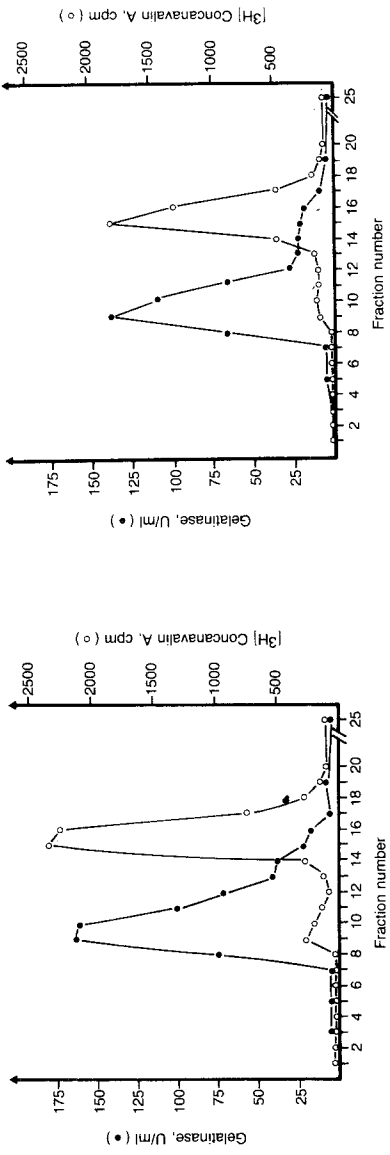

Fig. 3. Subcellular localization of NADPH-oxidase activity and biochemical markers in 4°C controls and LTB_4-stimulated neutrophils: 1.6×10^9 neutrophils were divided and one-half were incubated at 37°C and 10 min in the presence of 10^{-7} M LTB_4. The other half were kept on ice. After incubation, the cells were disrupted by nitrogen cavitation and the postnuclear supernatants layered on a two-step Percoll density gradients and centrifuged: 1.5-ml fractions were collected from the bottom. The left panel shows results of assays of neutrophil constituents in control samples. The right column shows results of the same markers in samples from stimulated cells. The peak values for myeloperoxidase (azurophil granules) and vitamin B_{12}-binding protein (specific granules) are indicated by closed and open triangles respectively. Note that NADPH-oxidase is measured in the presence of added cytosolic factors and SDS. From Bjerrum and Borregaard (1988).

(including both the plasma membrane proper and the membrane of granules that has fused with the plasma membrane during exocytosis) are activated during stimulation of the cells? The existence of this paradox may be denied by arguing that no direct evidence exists which shows that NADPH-oxidase generated in the cell-free system has any relation to NADPH-oxidase generated by intact cells. A more constructive way of thinking may identify the clue to this paradox as the activator of NADPH-oxidase. SDS and arachidonate are generally used as activators in the cell-free system. Protein kinase C has been shown to be capable of activating NADPH-oxidase in the cell-free system also (Cox *et al.*, 1985), and much indirect evidence indicates that the activator employed by the intact cell is protein kinase C (Tauber, 1987).

Protein kinase C is localized in the cytosol in unactivated cells and translocates to membranes when diacylglycerols emerge as a result of phospholipase C-mediated lysis of phosphatidyl inositols (Nishizuka, 1984). It is likely that diacylglycerols appear only in the plasma membrane. This would explain why only the NADPH-oxidase components present in the plasma membrane become activated. We have demonstrated (Christiansen and Borregaard, submitted) that agents like oleoylacetylglycerol and PMA, which mimic diacylglycerols in their ability to anchor protein kinase C to membranes, will cause translocation of protein kinase C to the plasma membrane, but not to the membrane of specific granules or azurophil granules when cytosol, as a source of protein kinase C, is mixed with isolated granules and plasma membranes in the presence of these agents. This indicates that receptors for protein kinase C, in addition to diacylglycerols, exist in the plasma membrane, but not in granule membranes except for membranes of the secretory granules. It should be stressed that it has not been definitely proven that protein kinase C is necessary for activation of NADPH-oxidase in intact cells by all stimuli (Tauber, 1987). Some evidence indicates that NADPH-oxidase may be activated both in intact cells and in cell-free systems without the participation of protein kinase C (Gerard *et al.*, 1986; Curnutte *et al.*, 1987a; Babior *et al.*, 1988).

Another possibility is that the so-called cytosolic factors may not be evenly distributed throughout the cytoplasm. These factors may be loosely attached to the plasma membrane in the unactivated cell and therefore only able to become engaged with the membrane-bound part of the NADPH-oxidase present in the plasma membrane.

These questions should be answered when all factors that are necessary and sufficient for assembly of NADPH-oxidase are identified and their subcellular localization in the intact cell determined by immunocytochemistry.

Acknowledgments

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